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(54) **PLANTS HAVING IMPROVED SEED YIELD AND METHOD FOR MAKING THE SAME**

PFLANZEN MIT GESTEIGERTEM SAATERTRAG UND VERFAHREN ZU DEREN HERSTELLUNG

PLANTES PRESENTANTS UN RENDEMENT D'ENSEMENCEMENT ACCRU ET PROCEDE DE PREPARATION CORRESPONDANT

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(56) References cited:
WO-A-02/50292 WO-A-98/42851

- **RIOU-KHAMLICHI C ET AL: "Cytokinin activation of Arabidopsis cell division through a D-type cyclin" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 283, no. 5407, 5 March 1999 (1999-03-05), pages 1541-1544, XP002134537 ISSN: 0036-8075**
- **DATABASE UNIPROT [Online] 1 May 2000 (2000-05-01), XP002292516 retrieved from EBI Database accession no. Q9SN11 -& DATABASE EMBL [Online] 9 December 2001 (2001-12-09), XP002292520 retrieved from EBI Database accession no. AY063729**
- **DEWITTE WALTER ET AL: "Altered cell cycle distribution, hyperplasia, and inhibited differentiation in Arabidopsis caused by the D-type cyclin CYCD3." PLANT CELL, vol. 15, no. 1, January 2003 (2003-01), pages 79-92, XP002292514 ISSN: 1040-4651 cited in the application**
- **LEE YI ET AL: "Expansins: Ever-expanding numbers and functions" CURRENT OPINION IN PLANT BIOLOGY, vol. 4, no. 6, December 2001 (2001-12), pages 527-532, XP002292515 ISSN: 1369-5266**
- **SWAMINATHAN KANKSHITA ET AL: "An enhancer trap line associated with a D-class cyclin gene in Arabidopsis" PLANT PHYSIOLOGY (ROCKVILLE), vol. 124, no. 4, December 2000 (2000-12), pages 1658-1667, XP002334496 ISSN: 0032-0889**

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Description

[0001] The present invention relates generally to the field of molecular biology and concerns a method for improving plant seed yield relative to that of corresponding wild type plants. More specifically, the present invention concerns a method for improving seed yield, by introducing into a plant a nucleic acid encoding a cyclin D3, which nucleic acid is under the control of a promoter preferentially expressed in the cell expansion zone of shoots. The present invention also concerns plants comprising an isolated cyclin D3 nucleic acid under the control of a promoter preferentially expressed in the cell expansion zone of shoots, which plants have improved seed yield relative to corresponding wild type plants. The invention also concerns constructs for use in the methods according to the invention.

[0002] The ever-increasing world population and the dwindling supply of arable land available for agriculture fuel research towards improving the efficiency of agriculture. Conventional means for crop and horticultural improvements utilise selective breeding techniques to identify plants having desirable characteristics. However, such selective breeding techniques have several drawbacks, namely that these techniques are typically labour intensive and result in plants that often contain heterogeneous genetic components that may not always result in the desirable trait being passed on from parent plants. Advances in molecular biology have allowed mankind to modify the germplasm of animals and plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has the capacity to deliver crops or plants having various improved economic, agronomic or horticultural traits. A trait of particular economic interest is yield, Yield is normally defined as the measurable produce of economic value from a crop. This may be defined in terms of quantity and/or quality. Yield is directly dependent on several factors, for example, the number and size of the organs, plant architecture (for example, the number of branches), seed production and more. Root development, nutrient uptake and stress tolerance are also important factors in determining yield. Crop yield may be increased by optimizing one of the abovementioned factors, which may be done by modifying the inherent growth mechanisms of a plant.

[0003] The inherent growth mechanisms of a plant reside in a highly ordered sequence of events collectively known as the 'cell cycle'. Progression through the cell cycle is fundamental to the growth and development of all multicellular organisms and is crucial to cell proliferation. The major components of the cell cycle are highly conserved in yeast, mammals, and plants. The cell cycle is typically divided into the following sequential phases: G0 - G1 - S - G2 - M. DNA replication or synthesis generally takes place during the S phase ("S" is for DNA synthesis) and mitotic segregation of the chromosomes occurs during the M phase (the "M" is for mitosis), with intervening gap phases, G1 (during which cells grow before DNA replication) and G2 (a period after DNA replication during which the cell prepares for division). Cell division is completed after cytokinesis, the last step of the M phase. Cells that have exited the cell cycle and that have become quiescent are said to be in the G0 phase. Cells in this phase can be stimulated to renter the cell cycle at the G1 phase. The "G" in G1, G2 and G0 stands for "gap". Completion of the cell cycle process allows each daughter cell during cell division to receive a full copy of the parental genome.

[0004] Cell division is controlled by two principal cell cycle events, namely initiation of DNA synthesis and initiation of mitosis. Each transition to each of these key events is controlled by a checkpoint represented by specific protein complexes (involved in DNA replication and division). The expression of genes necessary for DNA synthesis at the G1/S boundary is regulated by the E2F family of transcription factors in mammals and plant cells (La Thangue, 1994; Muller *et al.,* 2001; De Veylder *et al.,* 2002). Entry into the cell cycle is regulated/triggered by an E2F/Rb complex that integrates signals and allows activation of transcription of cell cycle genes. The transition between the different phases of the cell cycle, and therefore progression through the cell cycle, is driven by the formation and activation of different heterodimeric serine/threonine protein kinases, generally referred to as cyclin-dependent kinases (CDK). A prerequisite for activity of these kinases is the physical association with a specific cyclin, the timing of activation being largely dependent upon cyclin expression. Cyclin binding induces conformational changes in the N-terminal lobe of the associating CDK and contributes to the localisation and substrate specificity of the complex. Monomeric CDKs are activated when they are associated with cyclins and thus have a kinase activity. Cyclin protein levels fluctuate in the cell cycle and therefore represent a major factor in determining timing of CDK activation. The periodic activation of these complexes containing cyclins and CDK during cell cycle mediates the temporal regulation of cell-cycle transitions (checkpoints).

[0005] Cyclins can be grouped into mitotic cyclins (designated A- and B-type cyclins in higher eukaryotes and CLBs in budding yeast) and G1-specific cyclins (designated D-type cyclins in mammals and CLNs in budding yeast). H-type cyclins regulate the activity of the CAKs (CDK-activating kinases). All four types of cyclins known in plants were identified mostly by analogy to their human counterparts. In *Arabidopsis,* ten A-type, nine B-type, ten D-type and one H-type cyclin have been described (Vandepoele *et al.,* 2002).

[0006] The 10 D-type cyclins are subdivided into seven subclasses, D1 to D7, which reflect their lack of high sequence similarity to each other, which is in contrast to the A-type and B-type cyclins.

[0007] Only the D3 and D4 subclasses have different members, respectively three and two. Redundancy of the D3-type cyclins has been proposed previously as an explanation for the failure to observe mutant phenotypes upon knocking out of a single D3-type cyclin (Swaminathan *et al.,* 2000). The two D3-type cyclins are linked via a recent segmental

duplication, which suggests that these are functionally redundant. A similar hypothesis could hold for D4-type cyclins, because two out of three are located in a duplicated block.

[0008] The much larger divergence seen for D-type cyclins compared with A- and B-type cyclins might reflect the presumed role of D-type cyclins in integrating developmental signals and environmental cues into the cell cycle. For example, D3-type cyclins have been shown to respond to plant hormones, such as cytokinins and brassinosteroids, whereas CYCD2 and CYCD4 are activated earlier in G1 and react to sugar availability (for review, see Stals and Inzé, 2001).

[0009] Overexpression of the CYCD2;1 gene in tobacco was reported to increase cell division and increase overall plant growth rate with no morphological alterations (Cockcroft *et al.*, 2000).

[0010] Overexpression in *Arabidopsis* of the CYCD3;1 gene under the control of a CaMV 35S promoter was reported to give plants with enlarged cotyledons, a dramatically reduced final plant size and distorted development. At a cellular level, cells are pushed from G1, causing ectopic cell divisions in both meristematic regions and in regions in which cell division normally is absent or limited. This increase of cell numbers is coupled to a decrease in cell size (Dewitte *et al.,* 2003).

[0011] The ability to more accurately influence the cell cycle of a plant, and to thereby more accurately modify various growth characteristics of a plant, would have many applications in areas such as crop enhancement, plant breeding, in the production of ornamental plants, aboriculture, horticulture, forestry, the production of algae for use in bioreactors (for the biotechnological production of substances such as pharmaceuticals, antibodies, or vaccines, or for the bioconversion of organic waste) and other such areas.

[0012] It is an object of the present invention to overcome some of the problems associated with the prior art expression of cyclin D3 in plants.

[0013] It has now been found that introducing into a plant a nucleic acid encoding a cyclin D3 under the control of a promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of shoots gives plants having improved seed yield. Therefore according to the present invention there is provided a method for improving seed yield in a plant, comprising introducing into a plant a nucleic acid encoding a cyclin D3 under the control of a promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of shoots.

[0014] The term "increased yield" as defined herein is taken to mean an increase in any one or more of the following, each relative to corresponding wild type plants; (i) increased biomass (weight) of one or more aboveground (harvestable) parts of a plant; (ii) increased seed yield, which may result from an increase in the biomass of the seed (seed weight) and which may be an increase in the seed weight per plant or on an individual seed basis, and which increase in seed weight may be due to altered seed dimensions, such as seed length and/or seed width and/or seed area; (iii) increased number of (filled) seeds; (iv) increased seed size, which may also influence the composition of seeds; (v) increased seed volume, which may also influence the composition of seeds; (vi) increased harvest index, which is expressed as a ratio of the yield of harvestable parts, such as seeds, over the total biomass; and (vii) increased thousand kernel weight (TKW), which is extrapolated from the number of filled seeds counted and their total weight. An increased TKW may result from an increased seed size and/or seed density.

[0015] Taking corn as an example, a yield increase may be manifested as one or more of the following: increase in the number of plants per hectare or acre, an increase in the number of ears per plant, an increase in the number of rows, number of kernels per row, kernel weight, thousand kernel weight, ear length/diameter, among others. Taking rice as an example, a yield increase may be manifested by an increase in one or more of the following: number of plants per hectare or acre, number of panicles per plant, number of spikelets per panicle, number of flowers per panicle, increase in the seed filling rate, increase in thousand kernel weight, among others. An increase in yield may also result in modified architecture, or may occur as a result of modified architecture.

[0016] According to a preferred feature of the present invention, performance of the methods of the invention result in plants having improved seed yield which is manifested by at least one of: increased total seed number, increased number of (filled) seeds, increased seed weight and increased harvest index, each relative to corresponding wild type plants. Therefore, according to the present invention, there is provided a method for increasing plant yield, particularly seed yield, which method comprises introducing into a plant a nucleic acid encoding a cyclin D3 under the control of a promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of shoots.

[0017] Performance of the methods of the invention give improved seed yield in plants whether the plant (having introduced therein a cyclin D3 gene under the control of a promoter capable of preferentially expressing the nucleic acid in the cell expanion zone of shoots) is under non-stress conditions or whether the plant is exposed to various stresses compared to control plants. Plants typically respond to exposure to stress by growing more slowly. In conditions of severe stress, the plant may even stop growing altogether. Mild stress on the other hand is defined herein as being any stress to which a plant is exposed which does not result in the plant ceasing to grow altogether. Due to advances in agricultural practices (irrigation, fertilization, pesticide treatments) severe stresses are not often encountered in cultivated crop plants. As a consequence, the compromised growth induced by mild stress is often an undesirable feature for agriculture. Mild stresses are the typical stresses to which a plant may be exposed. These stresses may be the everyday biotic and/or

abiotic (environmental) stresses to which a plant is exposed. Typical abiotic or environmental stresses include temperature stresses caused by atypical hot or cold/freezing temperatures; salt stress; water stress (drought or excess water). Abiotic stresses may also be caused by chemicals. Biotic stresses are typically those stresses caused by pathogens, such as bacteria, viruses, fungi and insects.

[0018] Further advantageously, the methods of the may be performed on any plant.
The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, leaves, roots (including tubers), plant cells, tissues and organs, wherein each of the aforementioned preferably comprise the gene of interest. The term "plant" also encompasses suspension cultures, embryos, meristematic regions, callus tissue, gametophytes, sporophytes, pollen, and microspores, again wherein each of the aforementioned preferably comprise the gene of interest.

[0019] Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily Viridiplantae, in particular monocotyledonous and dicotyledonous plants including fodder or forage legumes, ornamental plants, food crops, trees or shrubs selected from the list comprising *Acacia* spp.,Acer spp., *Actinidia* spp., *Aesculus* spp., *Agathis australis, Albizia amara, Alsophila tricolor, Andropogon* spp., *Arachis* spp, *Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula* spp., *Brassica* spp., *Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra* spp, *Camellia sinensis, Canna indica, Capsicum* spp., Cassia spp., *Centroema pubescens, Chaenomeles* spp., *Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus* spp., *Cucumis* spp., *Cupressus spp., Cyathea dealbeta, Cydonia oblonga, Cryptomeria japonica, Cymbopogon* spp., *Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium* spp., *Dicksonia squarosa,*

[0020] *Diheteropogon amplectens, Dioclea* spp, *Dolichos* spp., *Dorycnium rectum, Echinochloa pyramidalis, Ehrartia* spp., *Eleusine coracana, Eragrestis* spp., *Erythrina* spp., *Eucalyptus* spp., *Euclea schimperi, Eulalia villosa, Fagopyrum* spp., *Feijoa sellowiana, Fragaria* spp., *Flemingia* spp, *Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia* spp, *Gossypium hirsutum, Grevillea* spp., *Guibourtia coleosperma, Hedysarum* spp., *Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incamata, Iris* spp., *Leptarrhena pyrolifolia, Lespediza* spp., *Lettuca* spp., *Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus* spp., *Macrotyloma axillare, Malus* spp., *Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum* spp., *Onobrychis* spp., *Omithopus* spp., *Oryza* spp., *Peltophorum africanum, Pennisetum* spp., *Persea gratissima, Petunia* spp., *Phaseolus* spp., *Phoenix canariensis, Phormium cookianum, Photinia* spp., *Picea glauca, Pinus* spp., *Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus* spp., *Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus* spp., *Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes* spp., *Robinia pseudoacacia, Rosa* spp., *Rubus* spp., *Salix* spp., *Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia* spp., *Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi* spp, *Taxodium distichum, Themeda triandra, Trifolium* spp., *Triticum* spp., *Tsuga heterophylla, Vaccinium* spp., *Vicia* spp., *Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays,* amaranth, artichoke, asparagus, broccoli, Brussels sprouts, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, strawberry, sugar beet, sugar cane, sunflower, tomato, squash, tea and algae, amongst others. According to a preferred embodiment of the present invention, the plant is a crop plant such as soybean, sunflower, canola, alfalfa, rapeseed, cotton, tomato, potato or tobacco. Further preferably, the plant is a monocotyledonous plant, such as sugarcane. More preferably the plant is a cereal, such as rice, maize, wheat, barley, millet, rye, oats or sorghum.

[0021] A cyclin D3 may be identified using different methods. For example, the query protein sequence may be blasted (for example, using blast default parameters for the gap opening penalty and the gap extension penalty) against a translated *Arabidopsis* nucleic acid sequence database. In the case where the query sequence is a cyclin D3, the first hit from the blast result will be an *Arabidopsis* cyclin D3. Another method for identifying a cyclin D3 is by aligning the query sequence with known cyclin D3 protein sequences, using for example the AlignX program from Vector NTI suite (InforMax, Bethesda, MD). Multiple alignments may then be carried out with a gap opening penalty of 10 and a gap extension of 0.01. Minor manual editing of the alignment may also be necessary in order to better position some conserved regions. If the query sequence is a cyclin D3, it will align with the known cyclin D3 sequences.

[0022] A "cyclin D3" as defined herein refers to any amino acid sequence which, when used in the construction of a cyclin or cyclin D phylogenetic tree, such as the one depicted in Fig. 1, falls into a group which includes cyclin D3s (and not other D-type cyclins, such as cyclin D1, D2, D4, D5, D6 and D7). Reference herein to a nucleic acid encoding a cyclin D3 is to a nucleic acid encoding a cyclin D3 amino acid as defined above.

[0023] A person skilled in the art could readily determine whether any amino acid sequence in question falls within the aforementioned definition using known techniques and software for the making of such a phylogenetic tree, such as a GCG, EBI or CLUSTAL package, using default parameters. Upon construction of such a phylogenetic tree, sequences clustering in the cyclin D3 group will be considered to fall within the definition of a "cyclin D3". Nucleic acids encoding

such sequences will be useful in performing the methods of the invention.

[0024] Cyclin D3s typically have the ability to bind and activate plant CDKs and Rb. In addition, cyclin D3s may comprise one or more and preferably all of the following: (i) a cyclin box; (ii) an LxCxE motif within the first 40 or so amino acids (which is characteristic of most cyclin Ds); and (iii) one or more and preferably all of the conserved regions identified by the boxes shown in Figure 2 (as shown in Figure 2, one mismatch within the boxes is allowed).

[0025] Examples of nucleic acids encoding cyclin D3s falling under the aforementioned definition of a cyclin D3 are given in Table 1 below. The cyclin D3-encoding nucleic acids shown in the table may be useful in performing the methods of the invention, i.e. to obtain plants having improved seed yield relative to corresponding wild type plants by introducing and expressing any one of these nucleic acids under the control of a promoter capable of preferentially expressing the nucleic acids in the cell expansion zone of a shoot. The nucleic acid encoding a cyclin D3 is preferably the nucleic acid represented by SEQ ID NO: 1 or is a functional variant of SEQ ID NO: 1, as described hereinafter.

Table 1: Examples of cyclin D3-encoding nucleic acids

Name	NCBI nucleic acid accession number	Source
Antma_cycD3a	AJ250397	Antirrhinum majus
Antma_cycD3b	AJ250398	Antirrhinum majus
Arath_CYCD3_1	NM_119579.2	Arabidopsis thaliana
Arath_CYCD3_2	NM_126126.2	Arabidopsis thaliana
Arath_CYCD3_3	NM_114867.2	Arabidopsis thaliana
Eupes_cycD3 like	AY340588	Euphorbia esula
Eupes_cycD3;1	AY340589	Euphorbia esula
Helan_cycD3	AY033440	Helianthus annuus
Heltu_cycD3;	AY063461	Helianthus tuberosus
Lagsi_cycD3:1	AF519810	Lagenaria siceraria
Lagsi_cycD3:2	AF519811	Lagenaria siceraria
Lyces_cycD3;	AJ002588	Lycopersicum esculentum
Lyces_cycD3;2	AJ002589	Lycopersicum esculentum
Lyces_cycD3,3	AJ002590	Lycopersicum esculentum
Medsa_cycD3	X88864	Medicago sativa
Nicta_cycD3;1	AJ011893	Nicotiana tabacum
Nicta_cycD3;2	AJ011894	Nicotiana tabacum
Nicta_cycD3;3	AB015222	Nicotiana tabacum
Orysa_cycD3-like	AK103499.1	Oryza sativa
Pissa_CycD3	AB008188	Pisum sativum
Popal_cycD3	AY230139	Populus alba
Poptr_cycD3	AF181993	Populus tremula x Populus tremuloides

[0026] According to the invention, enhanced or increased expression of the cyclin D3 nucleic acid in shoots is envisaged. Methods for obtaining enhanced or increased expression of genes or gene products is well documented in the art and include, for example, overexpression driven by promoters, the use of transcription enhancers or translation enhancers.

[0027] The nucleic acid encoding a cyclin D3 is operably linked to a promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of shoots. An example of such a promoter is a promoter having a comparable expression profile to the beta expansin promoter. It should be clear that the applicability of the present invention is not restricted to the cyclin D3 represented by SEQ ID NO: 1, nor is the applicability of the invention restricted to the use of the beta-expansin promoter in the methods of the invention.

[0028] The nucleic acid encoding a cyclin D3 may be derived from any source. The nucleic acid/gene encoding a

cyclin D3 may be isolated from a microbial source, such as yeast or fungi, or from a plant, algae or animal (including human) source. This nucleic acid may be modified from its native form in composition and/or genomic environment through deliberate human manipulation. The nucleic acid is preferably a homologous nucleic acid, i.e. a nucleic acid obtained from a plant, whether from the same plant species in which it is to be introduced or whether from a different plant species. The nucleic acid may be isolated from a dicotyledonous species, preferably from the family *Brassicaceae,* further preferably from *Arabidopsis thaliana.* More preferably, the cyclin D3 isolated from *Arabidopsis thaliana* is a D3-type cyclin, such as a cyclin D3;1, cyclin D3;2 or a cyclin D3;3. Most preferably, the cyclin D3 is cyclin D3;3 from *Arabidopsis thaliana,* particularly the nucleic acid sequence as represented by SEQ ID NO: 1 and the amino acid sequence as represented by SEQ ID NO: 2.

[0029] The sequence represented by SEQ ID NO: 1 depicts a cyclin D3;3 from *Arabidopsis thaliana,* with SEQ ID NO: 2 being the corresponding amino acid sequence. Advantageously, the applicability of the present invention is not restricted to the use of a cyclin D3;3 from *Arabidopsis* as represented by SEQ ID NO: 1. The methods according to the present invention may also be practised using functional variants of a cyclin D3-encoding nucleic acid or using functional variants of the encoded polypeptide. Especially useful in the methods of the invention are functional variants of the nucleic acid represented by SEQ ID NO: 1 or functional variants of the amino acid represented by SEQ ID NO: 2.

[0030] The term "functional variant" as defined herein is a cyclin D3 which retains the ability to bind and activate a plant CDK (see for example Healy et al. (2001, J. Biol. Chem. 276(10): 7041-7047)). Functional variants will in most cases also complement yeast mutants deficient of CLNs (the term for D-type cyclins in yeast), see for example, Swaminathan et al. (2000, Plant Phys 124: 1658-1667), particularly page 1663. The functional variant is a cyclin D3 in the sense that it is an amino acid sequence which, when used in the construction of a cyclin or cyclin D phylogenetic tree, such as the one depicted in Fig. 1, falls into a group which includes cyclin D3s (and not other D-type cyclins, such as cyclin D1, D2, D4, D5, D6 and D7). Reference herein to a nucleic acid encoding a cyclin D3 is to a nucleic acid encoding a cyclin D3 amino acid as defined above. In addition, the functional variant may comprise one or more and preferably all of the following: (i) a cyclin box; (ii) an LxCxE motif within the first 40 or so amino acids (which is characteristic of most cyclin Ds); and (iii) one or more and preferably all of the conserved regions identified by the boxes shown in Figure 2 (as shown in Figure 2, one mismatch within the boxes is allowed). Furthermore, a person skilled in the art may also readily determine whether a particular cyclin D3 is a functional variant (in the sense of whether it is able to improve plant seed yield) by simply substituting the sequence described in the Examples section below with the variant to be tested for function.

[0031] Suitable functional variant nucleic acid and amino acid sequences useful in practising the methods according to the invention, include:

(i) Portions of a cyclin D3-encoding nucleic acid, preferably a portion of a cyclin D3-encoding nucleic acid as represented by the sequence of SEQ ID NO: 1;
(ii) Alternative splice variants of a cyclin D3-encoding nucleic acid, preferably an alternative splice variant of a cyclin D3-encoding nucleic acid as represented by the sequence of SEQ ID NO: 1;
(iii) Allelic variants of a cyclin D3-encoding nucleic acid, preferably an allelic variant of a cyclin D3-encoding nucleic acid as represented by the sequence of SEQ ID NO: 1; and

[0032] Homologues, derivatives and active fragments of a cyclin D3 amino acid sequence, preferably a cyclin D3 as represented by the sequence of SEQ ID NO: 2.

[0033] It will be apparent to a person skilled in the art that the use of a full length cyclin D3-encoding DNA sequence would not be a prerequisite to carrying out the methods according to the invention. The methods according to the invention may advantageously be practised using functional portions of a cyclin D3-encoding DNA/nucleic acid, particularly a functional portion of a cyclin D3-encoding nucleic acid as represented by SEQ ID NO: 1. A portion refers to a piece of DNA derived or prepared from an original (larger) DNA molecule. A portion may be prepared, for example, by making one or more deletions to, for example, the nucleic acid sequence of SEQ ID NO: 1 using techniques well known in the art.

[0034] Therefore according to the invention, there is provided, a method for improving plant seed yield, comprising introducing into a plant a functional portion of a nucleic acid as represented by SEQ ID NO: 1. which functional portion is under the control of a promoter capable of preferentially expressing the functional portion in the cell expanion zone of shoots,

[0035] Another functional variant useful in the methods of the invention is an alternative splice variant of a cyclin D3-encoding nucleic acid, particularly an alternative splice variant of a cyclin D3-encoding nucleic acid as represented by the sequence of SEQ ID NO: 1. The term "alternative splice variant" as used herein encompasses variants of a nucleic acid sequence in which selected introns and/or exons have been excised, replaced or added. Such variants will be ones in which the biological activity of the protein remains unaffected, which can be achieved by selectively retaining functional segments of the protein. Such splice variants may be found in nature or may be manmade. Methods for making such splice variants are well known in the art.

[0036] Therefore, the invention also provides a method for improving seed yield in plants, comprising introducing into a plant an alternative splice variant of a cyclin D3-encoding nucleic acid, particularly an alternative splice variant of a cyclin D3-encoding nucleic acid as represented by SEQ ID NO: 1, which alternative splice variant is under the control of a promoter capable of preferentially expressing the splice variant in the cell expansion zone of shoots.

[0037] Another variant useful in the methods of the invention is an allelic variant of a cyclin D3-encoding nucleic acid, particularly an allelic variant of a cyclin D3-encoding nucleic acid as represented by the sequence of SEQ ID NO: 1. Allelic variants exist in nature and encompassed within the methods of the present invention is the use of these natural alleles. Allelic variants encompass Single Nucleotide Polymorphisms (SNPs), as well as Small Insertion/Deletion Polymorphisms (INDELs). The size of INDELs is usually less than 100 bp. SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms.

[0038] Therefore, the invention also provides a method for improving seed yield in plants, comprising introducing into a plant an allelic variant of a cyclin D3-encoding nucleic acid, particularly an allelic variant of a cyclin D3-encoding nucleic acid as represented by SEQ ID NO: 1, which allelic variant is under the control of a promoter capable of preferentially expressing the allelic variant in the cell expansion zone of shoots.

[0039] Further advantageously, the methods according to the present invention may also be practised using homologues, derivatives or active fragments of a cyclin D3, preferably using homologues, derivatives or active fragments of a cyclin D3 as represented by SEQ ID NO: 2. Nucleic acids encoding homologues, derivatives or active fragments of an amino acid as represented by SEQ ID NO: 2 may readily be determined using routine techniques well known to persons skilled in the art.

[0040] "Homologues" of a protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified protein in question and having similar biological and functional activity as the unmodified protein from which they are derived. To produce such homologues, amino acids of the protein may be replaced by other amino acids having similar properties (such as similar hydrophobicity, hydrophilicity, antigenicity, propensity to form or break α-helical structures or β-sheet structures). Conservative substitution tables are well known in the art (see for example Creighton (1984) Proteins. W.H. Freeman and Company).

[0041] The homologues useful in the method according to the invention are those falling under the definition of a functional variant, i.e. having the ability to bind and activate a plant CDK and being a cyclin D3, as defined hereinabove. Additionally, the homologues may be characterised in terms of having in increasing order of preference at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% sequence identity to the amino acid sequence as represented by SEQ ID NO: 2 (see Fig 3 which illustrates that cyclin D3s have a low percentage identity to each other, but nevertheless 30% identity to SEQ ID NO: 2 is sufficient to identify other cyclin D3s). In addition, the homologue may comprise one or more and preferably all of the following: (i) acyclin box; (ii) an LxCxE motif within the first 40 or so amino acids (which is characteristic of most cyclin Ds); and (iii) one or more and preferably all of the conserved regions identified by the boxes shown in Figure 2 (as shown in Figure 2, one mismatch within the boxes is allowed).

[0042] Also encompassed by the term "homologues" are two special forms of homology, which include orthologous sequences and paralogous sequences, which encompass evolutionary concepts used to describe ancestral relationships of genes. The term "paralogous" relates to gene-duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship.

[0043] Othologues in, for example, monocot plant species may easily be found by performing a so-called reciprocal blast search. This may be done by a first blast involving blasting the sequence in question (for example, SEQ ID NO: 1 or SEQ ID NO: 2) against any sequence database, such as the publicly available NCBI database which may be found at: http://www.ncbi.nlm.nih.gov. If orthologues in rice were sought, the sequence in question would be blasted against, for example, the 28,469 full-length cDNA clones from *Oryza saliva* Nipponbare available at NCBI. BLASTn may be used when starting from nucleotides or TBLASTX when starting from the protein, with standard default values (expectation 10, alignment 50). The blast results may be filtered. The full-length sequences of either the filtered results or the non-filtered results are then blasted back (second blast) against the sequence in question (SEQ ID NO: 1 or 2). The results of the first and second blasts are then compared. In the case of large families, ClustalW is used followed by a neighbour joining tree to help visualize the clustering.

[0044] A homologue may be in the form of a "substitutional variant" of a protein, i.e. where at least one residue in an amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1 to 10 amino acid residues, and deletions will range from about 1 to 20 residues. Preferably, amino acid substitutions comprise conservative amino acid substitutions.

[0045] A homologue may also be in the form of an "insertional variant" of a protein, i.e. where one or more amino acid residues are introduced into a predetermined site in a protein. Insertions may comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino- or carboxy-terminal fusions, of the order of about 1 to 10 residues. Examples of amino- or carboxy-terminal fusion proteins or peptides include the binding domain or activation domain of

a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, (histidine)6-tag, glutathione S-transferase-tag, protein A, maltose-binding protein, dihydrofolate reductase, Tag·100 epitope, c-myc epitope, FLAG®-epitope, lacZ, CMP (calmodulin-binding peptide), HA epitope, protein C epitope and VSV epitope.

[0046] Homologues in the form of "deletion variants" of a protein are characterised by the removal of one or more amino acids from a protein.

[0047] Amino acid variants of a protein may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. Methods for the manipulation of DNA sequences to produce substitution, insertion or deletion variants of a protein are well known in the art. For example, techniques for making substitution mutations at predetermined sites, in DNA are well known to those skilled in the art and include M13 mutagenesis, T7-Gen *in vitro* mutagenesis (USB, Cleveland, OH), QuickChange Site Directed mutagenesis (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

[0048] "Derivatives" include peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise substitutions, deletions or additions of naturally and non-naturally occurring amino acid residues compared to the amino acid sequence of a naturaily-occurring form of the protein, for example, as presented in SEQ ID NO: 2. "Derivatives" of a protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise naturally occurring altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the polypeptide. A derivative may also comprise one or more non-amino acid substituents compared to the amino acid sequence from which it is derived, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence, such as a reporter molecule which is bound to facilitate its detection, and non-naturally occurring amino acid residues relative to the amino acid sequence of a naturally-occurring protein,

[0049] "Active fragments" of a cyclin D3 protein encompasses at least five contiguous amino acid residues of a protein, which residues retain similar biological and/or functional activity to the naturally occurring protein. In any case, "homologues, derivatives and active fragments" are those falling under the definition of "functional variant" as defined hereinabove.

[0050] Methods for the search and identification of cyclin D3 homologues would be well within the realm of a person skilled in the art. Methods for the alignment of sequences for comparison are well known in the art, such methods include GAP, BESTFIT, BLAST, FASTA, ALIGN X (from vector NTI) and TFASTA. GAP uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48: 443-453, 1970) to find the alignment of two complete sequences that maximises the number of matches and minimises the number of gaps. The BLAST algorithm calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information. Homologues suitable for use in the methods of the invention, i.e. those having at least 30% sequence identity to the amino acid sequence represented by SEQ ID NO: 2, may be identified by taking full length cyclin D3 protein sequences and using a similarity/identity matrix generator, such as MatGAT (Matrix Global Alignment Tool) which calculates the similarity and identity between every pair of sequences in a given data set without requiring pre-alignment of the data. The program performs a series of pairwise alignments using the Myers and Miller global alignment algorithm (with a gap opening penalty of 12, and a gap extension penalty of 2). It then calculates similarity and identity using, for example, Blosum 60 as scoring matrix, and then places the results in a distance matrix.

[0051] Therefore, the invention also provides a method for improving plant seed yield, comprising introducing into a plant a nucleic acid encoding a homologue, derivative or active fragment of a cyclin D3, such as a homologue, derivative or active fragment of a cyclin D3 as represented by SEQ ID NO: 2, which homologue, derivative or active fragment is under the control of a promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of shoots.

[0052] Genetic constructs and vectors facilitate introduction and/or expression of the nucleotide sequences useful in the methods according to the invention.

[0053] Therefore, there is provided a gene construct comprising;

(i) a cyclin D3-encoding nucleic acid or functional variant thereof, preferably a cyclin D3-encoding nucleic acid as represented by SEQ ID NO: 1 or a functional variant thereof (as defined hereinabove), which nucleic acid encodes a cyclin D3 polypeptide or functional variant thereof, preferably a cyclin D3 polypeptide as represented by SEQ ID NO: 2 or a functional variant thereof;

(ii) a promoter capable of preferentially expressing the nucleic acid of (i) in shoots, particularly in the cell expansion zone of vegetative (aboveground) shoots; and optionally

(iii) a transcription termination sequence.

[0054] Constructs useful in the methods according to the present invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The gene constructs may be inserted into vectors, which may

be commercially available, suitable for transforming into plants and suitable for expression of the gene of interest in the transformed cells.

[0055] Plants are transformed with a vector comprising the sequence of interest (i.e., a nucleic acid encoding a cyclin D3 or a functional variant thereof (as defined hereinbefore), for example a cyclin D3 encoding nucleic acid as represented by SEQ ID NO: 1 or a functional variant thereof (as defined hereinabove)). The sequence of interest is operably linked to a promoter capable of preferentially expressing the sequence of interest in shoots. The terms "regulatory element", "control sequence" and "promoter" are all used interchangeably herein and are to be taken in a broad context to refer to regulatory nucleic acid sequences capable of effecting expression of the sequences to which they are ligated. Encompassed by the aforementioned terms are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Also included within the term is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or -10 box transcriptional regulatory sequences. The term "regulatory element" also encompasses a synthetic fusion molecule or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

[0056] The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest.

[0057] The nucleic acid encoding a cyclin D3 or a functional variant thereof, such as a cyclin D3-encoding nucleic acid as represented by SEQ ID NO: 1 or a functional variant thereof, is operably linked to a promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of shoots. Preferably, the promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of shoots has a comparable expression profile to a beta-expansin promoter, for example as shown in Fig. 5. A person skilled in the art will readily be able to identify promoters having a comparable expression profile to a beta-expansin promoter using routine techniques. More specifically, the promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of a shoot, is capable of driving expression particularly in vegetative (aboveground) shoots. Most preferably, the promoter capable of preferentially expressing the nucleic acid in shoots is the beta-expansin promoter (SEQ ID NO: 3).

[0058] Optionally, one or more terminator sequences may also be used in the construct introduced into a plant. The term "terminator" encompasses a control sequence which is a DNA sequence at the end of a transcriptional unit which signals 3' processing and polyadenylation of a primary transcript and termination of transcription. Additional regulatory elements may include transcriptional as well as translational enhancers. Those skilled in the art will be aware of terminator and enhancer sequences which may be suitable for use in performing the invention. Such sequences would be known or may readily be obtained by a person skilled in the art.

[0059] The genetic constructs may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type. One example is when a genetic construct is required to be maintained in a bacterial cell as an episomal genetic element (e.g. plasmid or cosmid molecule). Preferred origins of replication include, but are not limited to, the f1-ori and colE1.

[0060] The genetic construct may optionally comprise a selectable marker gene. As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a nuclei acid construct of interest. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance, that introduce a new metabolic trait or that allow visual selection. Examples of selectable marker genes include genes conferring resistance to antibiotics (such as nptII that phosphorylates neomycin and kanamycin, or hpt, phosphorylating hygromycin), to herbicides (for example bar which provides resistance to Basta; aroA or gox providing resistance against glyphosate), or genes that provide a metabolic trait (such as manA that allows plants to use mannose as sole carbon source). Visual marker genes result in the formation of colour (for example β-glucuronidase, GUS), luminescence (such as luciferase) or fluorescence (Green Fluorescent Protein, GFP, and derivatives thereof).

[0061] The present invention also encompasses plants obtainable by the methods according to the present invention. The present invention therefore provides plants obtainable by the method according to the present invention, which plants comprise a nucleic acid encoding a cyclin D3 or functional variant thereof operably linked to a promoter capable of preferentially expressing the nucleic acid in the cell expension zone of a shoot.

[0062] The invention also provides a method for the production of transgenic plants having improved yield, comprising introduction into a plant of a cyclin D3-encoding nucleic or functional variant thereof, particularly a cyclin D3-encoding nucleic acid as represented by SEQ ID NO: 1 or a functional variant thereof (as defined hereinabove), which nucleic acid is operably linked to a promoter capable of preferentially expressing the nucleic acid in the cell expansion zone of a shoot.

[0063] More specifically, the present invention provides a method for the production of transgenic plants having improved yield relative to corresponding wild type plants, which method comprises:

(i) introducing into a plant or plant cell a cyclin D3-encoding nucleic acid or a functional variant thereof, preferably a cyclin D3-encoding nucleic acid as represented by SECT ID NO: 1 or a functional variant thereof, which nucleic acid or functional variant thereof encodes a cyclin D3 polypeptide or functional variant thereof, which polypeptide is preferably as represented by SEQ ID NO: 2 or is a functional variant thereof and which nucleic acid or functional variant thereof is under the control of a promoter capable of preferentially expressing the nucleic acid in shoots, particularly in the cell expansion zone of vegetative (aboveground) shoots;
(ii) cultivating the plant cell under conditions promoting regeneration and mature plant growth.

[0064] The nucleic acid or functional variant may be introduced directly into a plant cell or into the plant itself (including introduction into a tissue, organ or any other part of a plant). According to a preferred feature of the present invention, the nucleic acid is preferably introduced into a plant by transformation.

[0065] The term "introducing into a plant" refers principally to transformation of a plant with the particular nucleic acid in question (a cyclin D3-encoding nucleic acid or functional variant thereof), however the term also refers to other methods which result in the introduction into a plant of the particular nucleic acid in question, such as breeding techniques. Breeding techniques are well known to persons skilled in the art.

[0066] The term "transformation" as referred to herein encompasses the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem). The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid. Alternatively, it may be integrated into the host genome. The resulting transformed plant cell may then be used to regenerate a transformed plant in a manner known to persons skilled in the art.

[0067] Transformation of plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., 1882, Nature 296, 72-74; Negrutiu I. et al., June 1987, Plant Mol. Biol. 8, 363-373); electroporation of protoplasts (Shillito R.D. et al., 1985 Bio/Technol 3, 1099-1102); microinjection into plant material (Crossway A. et al., 1986, Mol. Gen Genet 202, 179-185); DNA or RNA-coated particle bombardment (Klein T.M. et al., 1987, Nature 327, 70) infection with (non-integrative) viruses and the like. Transgenic rice plants expressing a cyclin D3-encoding nucleic acid or functional variant thereof are preferably produced via *Agrobacterium*-mediated transformation using any of the well known methods for rice transformation, such as described in any of the following: published European patent application EP 1198985 A1, Aldemita and Hodges (Planta, 199, 612-617, 1996); Chan et al. (Plant Mol. Biol. 22 (3) 491-506, 1993), Hiei et al. (Plant J. 6 (2) 271-282, 1994), which disclosures are incorporated by reference herein as if fully set forth. In the case of com transformation, the preferred method is as described in either Ishida et al. (Nat. Biotechnol. 1996 Jun; 14(6): 745-50) or Frame et a/. (Plant Physiol. 2002 May; 129(1): 13-22), which disclosures are incorporated by reference herein as if fully set forth.

[0068] Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant-expressible genes co-transferred with the gene of interest, following which the transformed material is regenerated into a whole plant.

[0069] Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be monitored using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

[0070] The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformants, and the T2 plants further propagated through classical breeding techniques.

[0071] The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

[0072] The methods according to the invention may also be performed without introducing a nucleic acid encoding a cyclin D3 or functional variant thereof into a plant. This may be achieved by introducing a genetic modification, preferably in the locus of a cyclin D3-encoding gene, so as to allow for expression of the gene preferentially in shoots. The locus

of a gene as defined herein is taken to mean a genomic region, which includes the gene of interest and 10KB up- or down stream of the coding region.

[0073] The genetic modification may be introduced, for example, by any one (or more) of the following methods: T-DNA activation, TILLING, mutagenesis, homologous recombination or, as discussed hereinabove, by introducing and expressing in a plant (cell) a cyclin D3-encoding nucleic acid or functional variant thereof, the nucleic acid being under the control of a promoter capable of preferentially expressing that nucleic acid in shoots.

[0074] T-DNA activation tagging (Hayashi et al. Science (1992) 1350-1353) involves insertion of T-DNA usually containing a promoter (may also be a translation enhancer or an intron), in the genomic region of the gene of interest or 10KB up- or down stream of the coding region of a gene in a configuration such that the promoter directs expression of the targeted gene. Typically, regulation of expression of the targeted gene by its natural promoter is disrupted and the gene falls under the control of the newly introduced promoter. The promoter is typically embedded in a T-DNA. This T-DNA is randomly inserted into the plant genome, for example, through *Agrobacterium* infection and leads to overexpression of genes near to the inserted T-DNA. The resulting transgenic plants show dominant phenotypes due to overexpression of genes close to the introduced promoter. In order to achieve improved yield, the promoter to be introduced would be any promoter capable of preferentially expressing in shoots.

[0075] A genetic modification may also be introduced in the locus of a cyclin D3-encoding nucleic acid/gene using the technique of TILLING (Targeted Induced Local Lesions IN Genomes). This is a mutagenesis technology useful to generate and/or identify, and to eventually isolate mutagenised variants of a cyclin D3-encoding nucleic acid capable of exhibiting cyclin D3 biological activity. TILLING also allows selection of plants carrying such mutant variants. These mutant variants may even exhibit higher cyclin D3 activity than that exhibited by the gene in its natural form. TILLNG combines high-density mutagenesis with high-throughput screening methods. The steps typically followed in TILLING are: (a) EMS mutagenesis (Redei and Koncz, 1992; Feldmann *et al.,* 1994; Lightner and Caspar, 1998); (b) DNA preparation and pooling of individuals; (c) PCR amplification of a region of interest; (d) denaturation and annealing to allow formation of heteroduplexes; (e) DHPLC, where the presence of a heteroduplex in a pool is detected as an extra peak in the chromatogram; (f) identification of the mutant individual; and (g) sequencing of the mutant PCR product. Methods for TILLING are well known in the art (McCallum Nat Biotechnol; 2000 Apr, 18(4):455-7, reviewed by Stemple 2004 (TILLING-a high-throughput harvest for functional genomics. Nat Rev Genet. 2004 Feb;5(2):145-50.)).

[0076] Mutagenesis may be used to generate variants of cyclin D3-encoding nucleic acids. Methods for generating mutant variants are well known in the art.

[0077] TDNA activation, TILLING and site-directed mutagenesis are examples of technologies that enable the generation of novel alleles and cyclin D3 variants.

[0078] Homologous recombination allows introduction in a genome of a selected nucleic acid at a defined selected position. Homologous recombination is a standard technology used routinely in biological sciences for lower organisms such as yeast or the moss physcomitrella. Methods for performing homologous recombination in plants have been described not only for model plants (Offringa et al. Extrachromosomal homologous recombination and gene targeting in plant cells after Agrobacterium-mediated transformation. 1990 EMBO J. 1990 Oct; 9(10):3077-84) but also for crop plants, for example rice (Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S. Efficient gene targeting by homologous recombination in rice. Nat Biotechnol. 2002. Iida and Terada: A tale of two integrations, transgene and T-DNA: gene targeting by homologous recombination in rice. Curr Opin Biotechnol. 2004 Apr; 15(2):132-8). The nucleic acid to be targeted (which may be a cyclin D3-encoding nucleic acid or variant thereof as hereinbefore defined) need not be targeted to the locus of a cyclin D3-encoding gene, but may be introduced in, for example, regions of high expression. The nucleic acid to be targeted may be an improved allele used to replace the endogenous gene or may be introduced in addition to the endogenous gene.

[0079] The present invention clearly extends to any plant cell or plant produced by any of the methods described herein, and to all plant parts and propagules thereof. The present invention extends further to encompass the progeny of a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by any of the aforementioned methods, the only requirement being that progeny exhibit the same genotypic and/or phenotypic characteristic(s) as those produced in the parent by the methods according to the invention. The invention also includes host cells containing an isolated nucleic acid molecule encoding a cyclin D3 operably linked to a promoter capable of expressing the nucleic acid in the cell expansion zone of a shoot. Preferred host cells according to the invention are plant cells. The invention also extends to harvestable parts of a plant such as but not limited to seeds, leaves, fruits, flowers, stem cultures, rhizomes, tubers and bulbs.

[0080] The present invention also encompasses the use of nucleic acids encoding cyclin D3s and the use of cyclin D3 polypeptides.

[0081] One such use of course relates to the use of a cyclin D3-encoding nucleic acid, operably linked to a promoter capable of preferentially expressing the nucleic acid in shoots, in improving the seed yield of plants, in particular in increasing total seed number, increasing number of (filled) seeds, increasing seed weight and increasing harvest index, among others. The cyclin D3 -encoding nucleic acid or functional variant thereof are as defined hereinabove. Preferred

is a nucleic acid as represented by SEQ ID NO: 1, or a functional variant thereof as hereinbefore defined.

[0082] Nucleic acids encoding cyclin D3s and cyclin D3 polypeptides may also find use in breeding programmes. The cyclin D3 may be a nucleic acid as represented by SEQ ID NO: 1, or a functional variant thereof as hereinbefore defined; or the cyclin D3 may be an amino acid as represented by SEQ ID NO: 2 or a functional variant thereof as hereinbefore defined. For example, the cyclin D3-encoding nucleic acid or a part thereof may be on a chromosome (or a part thereof), preferably together with one or more related family members. In an example of such a breeding programme, a DNA marker is identified which may be genetically linked to a nucleic acid encoding a cyclin D3 protein in a plant, which gene may be a gene encoding the cyclin D3 protein itself or any other gene which may directly or indirectly influence expression of a gene encoding a cyclin D3 protein and/or activity of the cyclin D3 protein itself. This DNA marker may then be used in breeding programs to select plants having improved yield relative to corresponding wild type plants .

[0083] Allelic variants of a cyclin D3 may also be used in conventional breeding programmes, such as in marker-assisted breeding. Such breeding programmes sometimes require the introduction of allelic variations in the plants by mutagenic treatment of a plant. One suitable mutagenic method is EMS mutagenesis. Identification of allelic variants then takes place by, for example, PCR. This is followed by a selection step for selection of superior allelic variants of the sequence in question and which give improved seed yield in a plant relative to corresponding wild type plants. Selection is typically carried out by monitoring the seed yield in plants containing different allelic variants of the sequence in question, for example, different allelic variants of SEQ ID NO: 1. Monitoring yield can be done in a greenhouse or in the field. Further optional steps include crossing plants, in which the superior allelic variant was identified, with another plant. This could be used, for example, to make a combination of interesting phenotypic features.

[0084] Nucleic acids encoding cyclin D3s and cyclin D3 polypeptides may also find use as growth regulators. The cyclin D3 may be a nucleic acid as represented by SEQ ID NO: 1, or a functional variant thereof as hereinbefore defined; or the cyclin D3 may be an amino acid as represented by SEQ ID NO: 2 or a functional variant thereof as hereinbefore defined. Since these cyclin D3s are useful in improving seed yield in plants, the cyclin D3s would also be useful growth regulators, such as herbicides or growth stimulators. The present invention therefore provides a composition comprising a cyclin D3, together with a suitable carrier, diluent or excipient, for use as a growth regulator.

[0085] The methods according to the present invention result in plants having improved seed yield, as described hereinbefore. These advantageous characteristics may also be combined with other economically advantageous traits, such as further yield-enhancing traits, tolerance to various stresses, traits modifying various architectural features and/or biochemical and/or physiological features.

Description of figures

[0086] The present invention will now be described with reference to the following figures in which:

Fig. 1 is a multiple alignment prepared using ClustalW and default values, followed by average distance tree computation. The cyclin D3 cluster is shown.

Fig. 2 is an alignment of known cyclin D3 protein sequences. The sequences were aligned using AlignX program from Vector NTI suite (InforMax, Bethesda, MD). Multiple alignment was done with a gap opening penalty of 10 and a gap extension of 0.01. Minor manual editing was also carried out where necessary to better position some conserved regions. The line shown indicates the separation of cyclin D3s from other cyclins Ds. A number of motifs specific to cyclin D3s are boxed.

Fig. 3 is a similarity/identity matrix prepared using MatGAT (Matrix Global Alignment Tool) which calculates the similarity and identity between every pair of sequences in a given data set without requiring pre-alignment of the data. The program performs a series of pairwise alignments using the Myers and Miller global alignment algorithm (with a gap opening penalty of 12, and a gap extension penalty of 2). It then calculates similarity and identity using, for example, Blosum 60 as scoring matrix, and then places the results in a distance matrix. Sequence similarity is shown in the bottom half of the dividing line and sequence identity is shown in the top half of the dividing line. The sequence of SEQ ID NO: 2 is indicated as number 5 in the matrix. Sequences having at least 30% sequence identity to the sequence of SEQ ID NO: 2 encompass cyclin D3s.

Fig. 4 is a binary vector for expression in *Oryza sativa* of the *Arabidopsis thaliana* CyclinD3;3 gene under the control of the beta-expansin promoter.

Fig. 5 shows photographs of GUS expression driven by a beta expansin promoter. The photograph of the "C plant" is of a rice plant GUS stained when it had reached a size of about 5 cm. The photograph of the "B plant" is of a rice plant GUS stained when it had reached a size of about 10 cm. Promoters with comparable expression profiles may

also be useful in the methods of the invention.

Fig. 6 details examples of sequences useful in performing the methods according to the present invention.

Examples

[0087] The present invention will now be described with reference to the following examples, which are by way of illustration alone.

[0088] DNA manipulation: unless otherwise stated, recombinant DNA techniques are performed according to standard protocols described in (Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York) or in Volumes 1 and 2 of Ausubel et al. (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1: Gene Cloning

[0089] The *Arabidopsis* Cyclin D3;3 (internal reference CDS0018) was amplified by PCR using as template an *Arabidopsis thaliana* seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNAs were cloned into pCMV Sport 6.0. Average insert size of the bank was 1.5 kb and original number of clones was of $1.59x10^7$ cfu. Original titer was determined to be $9.6x10^5$ cfu/ml after first amplification of $6x10^{11}$ cfu/ml. After plasmid extraction, 200ng of template was used in a 50 μl PCR mix. Primers prm0360 (sense, start codon in bold, AttB1 site in italic: 5' *GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACA*ATGGCTTTAGAAGAGGAGGA 3') and prm0361 (reverse, complementary, stop codon in bold, AttB2 site in italic: 5' *GGGGACCACTTTGTACAAGAAAGCTGGGT*TTAGCGAGGACTACTATAAGCA 3'), which include the AttB sites for Gateway recombination, were used for PCR amplification. PCR was performed using Hifi Taq DNA polymerase in standard conditions. A PCR fragment of 1086 bp was amplified and purified also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment recombines *in vivo* with the pDONR201 plasmid to produce, according to the Gateway terminology, an "entry clone", p0443. Plasmid pDONR201 was purchased from Invitrogen, as part of the Gateway® technology.

Example 2: Vector Construction

[0090] The entry clone p0443 was subsequently used in an LR reaction with p3169, a destination vector used for *Oryza sativa* transformation. This vector contains as functional elements within the T-DNA borders: a plant selectable marker; a plant screenable marker; and a Gateway cassette intended for LR *in vivo* recombination with the sequence of interest already cloned in the entry clone. A beta-expansin promoter for expression in the expansion zone of vegetative (aboveground) shoots is located upstream of this Gateway cassette.

[0091] After the LR recombination step, the resulting expression vector (see Figure 4) was transformed into *Agrobacterium* strain LBA4404 and subsequently to *Oryza sativa* plants. Transformed rice plants were allowed to grow and were then examined for the parameters described in Example 3.

Example 3: Evaluation and Results

[0092] Approximately 15 to 20 independent T0 rice transformants were generated. The primary transformants were transferred from tissue culture chambers to a greenhouse for growing and harvest of T1 seed. 5 events, of which the T1 progeny segregated 3:1 for presence/absence of the transgene, were retained. For each of these events, approximately 10 T1 seedlings containing the transgene (hetero- and homo-zygotes), and approximately 10 T1 seedlings lacking the transgene (nullizygotes), were selected by monitoring visual marker expression. The best T1 events were further evaluated in the T2 generation following the same procedure as for the T1 generation, but with more individuals per event.

Statistical analysis: t-test and F-test

[0093] A two factor ANOVA (analysis of variants) was used as a statistical model for the overall evaluation of plant phenotypic characteristics. An F-test was carried out on all the parameters measured of all the plants of all the events transformed with the gene of the present invention. The F-test was carried out to check for an effect of the gene over all the transformation events and to verify for an .overall effect of the gene, also known as a global gene effect. The threshold for significance for a true global gene effect was set at a 5% probability level for the F-test. A significant F-test

value points to a gene effect, meaning that it is not only the presence or position of the gene that is causing the differences in phenotype.

3.1 Vegetative growth measurements:

[0094] The selected T1 plants (approximately 10 with the transgene and approximately 10 without the transgene) were transferred to a greenhouse. Each plant received a unique barcode label to link unambiguously the phenotyping data to the corresponding plant. The selected T1 plants were grown on soil in 10 cm diameter pots under the following environmental settings: photoperiod= 11.5 h, daylight intensity= 30,000 lux or more, daytime temperature= 28°C or higher, night time temperature= 22°C, relative humidity= 60-70%. Transgenic plants and the corresponding nullizygotes were grown side-by-side at random positions. From the stage of sowing until the stage of maturity each plant was passed several times through a digital imaging cabinet and imaged. At each time point digital images (2048x1536 pixels, 16 million colours) were taken of each plant from at least 6 different angles. The parameters described below were derived in an automated way from all the digital images of all the plants, using image analysis software.

3.1.1 Aboveground plant area

[0095] Plant aboveground area was determined by counting the total number of pixels from aboveground plant parts discriminated from the background. This value was averaged for the pictures taken on the same time point from the different angles and was converted to a physical surface value expressed in square mm by calibration. Experiments show that the aboveground plant area measured this way correlates with the biomass of plant parts above ground. The results of the T1 and T2 evaluation are shown in Table 1 below. The percentage difference between transgenic plants and corresponding nullizygotes is shown. The p value of the F test was significant in both the T1 and T2 evaluation indicating an overall effect of the presence of the transgene on aboveground area.

Table 2: Aboveground area

Aboveground area		
	% Difference	P value
T1 Overall	15	0.001
T2 Overall	18	0.0003

3.2 Seed-related parameter measurements

[0096] The mature primary panicles were harvested, bagged, barcode-labelled and then dried for three days in the oven at 37°C. The panicles were then threshed and all the seeds were collected and counted. The filled husks were separated from the empty ones using an airblowing device. The empty husks were discarded and the remaining fraction was counted again. The filled husks were weighed on an analytical balance. This procedure resulted in the set of seed-related parameters described below.

3.2.1 Total number of Seeds

[0097] Total seed number per plant was measured by counting the number of husks harvested from a plant. The results of the T1 and T2 evaluations are shown in Table 2 below. The percentage difference between transgenic plants and corresponding nullizygotes is shown. The p value of the F test was significant for both the T1 and T2 evaluation indicating that the presence of the transgene has a significant effect on the total number of seeds produced.

Table 3: Total Number of Seeds

Total number of seeds		
	% Difference	P value
T1 Overall	27	0.0039
T2 Overall	19	0.0096

3.2.2 Number of filled seeds

[0098] The number of filled seeds was determined by counting the number of filled husks that remained after the separation step. The results of the T1 and T2 evaluations are shown in Table 3 below. The percentage difference between transgenic plants and corresponding nullizygotes is shown. The p value of the F test was significant in both the T1 and T2 evaluations indicating that the presence of the transgene significantly increases the number of filled seeds produced.

Table 4: Number of Filled Seeds

Total number of filled seeds		
	% Difference	P value
T1 Overall	52	0.0002
T2 Overall	35	0.0007

3.2.3 Total seed weight

[0099] The total seed yield was measured by weighing all filled husks harvested from a plant. The results of the T1 and the T2 evaluations are shown in Table 4 below. The percentage difference between transgenic plants and corresponding nullizygotes is shown. The p value of the F test was significant for both the T1 and T2 evaluations indicating that the presence of the transgene significantly increases seed weight.

Table 5: Total Seed Weight

Total Seed Weight		
	% difference	P value
T1 Overall	42	0.0017
T2 Overall	38	0.0005

3.2.4 Harvest index of plants

[0100] The harvest index in the present invention is defined as the ratio between the total seed yield and the above ground area (mm^2), multiplied by a factor 10^6. The results of the T1 and T2 evaluation are shown in Table 5 below. The percentage difference between transgenic plants and corresponding nullizygotes is shown. The p value of the F test was significant for both the T1 and T2 evaluations indicating that the presence of the transgene significantly increases the harvest index.

Table 6: Harvest Index

Harvest index		
	% Difference	P value
T1 Overall	28	0.0022
T2 Overall	20	0.0086

Example 4: Comparative data pOleosin::cyclin D3;3

[0101] Plants containing the above construct were produced and evaluated using the same procedures as described above for pBeta-expansin::cyclin D3;3. The results of the T1 evaluation are shown in tables 6 to 8 below. The percentage difference between transgenic plants and corresponding nullizygotes is shown in each of the tables. The p value of the F test is also shown.

Table 7: Aboveground Area

Aboveground area		
	% Difference	P value
T1 Overall	-12	0.0083

[0102] The p value of the F test was significant indicating that the expression of the transgene driven by this promoter significantly decreases aboveground area.

Table 8: Total Seed Weight

Total Seed Weight		
	% difference	P value
T1 Overall	-15	0.0858

[0103] The results show that the total weight of the seeds of transgenic plants was lower than the total seed weight of corresponding nulllizygotes.

Table 9: Number of Filled Seeds

Number of Filled Seeds		
	% difference	P value
T1 Overall	-17	0.0572

[0104] The results show that the number of filled seeds of transgenic plants was lower than the number of filled seeds of corresponding nulllizygotes.

Example 5: GUS expression driven by beta expansin promoter

[0105] The beta-expansin promoter was cloned into the pDONR201 entry plasmid of the GatewayTM system (Life Technologies) using the "BP recombination reaction". The identity and base pair composition of the cloned insert was confirmed by sequencing and additionally, the resulting plasmid was tested via restriction digests.

[0106] In order to clone the promoter in front of a reporter gene, each entry clone was subsequently used in an "LR recombination reaction" (Gateway TM) with a destination vector. This destination vector was designed to operably link the promoter to the *Escherichia coli* beta-glucuronidase (GUS) gene via the substitution of the Gateway recombination cassette in front of the GUS gene. The resulting reporter vectors, comprising the promoter operably linked to GUS were are subsequently transformed into *Agrobacterium* strain LBA4044 and subsequently into rice plants using standard transformation techniques.

[0107] Transgenic rice plants were generated from transformed cells. Plant growth was performed under normal conditions.

[0108] The plants or plant parts to be tested were covered with 90% ice-cold acetone and incubated for 30 min at 4 °C. After 3 washes of 5 min with Tris buffer [15,76 g Trizma HCl (Sigma T3253) + 2,922 g NaCl in 1 litre bi-distilled water, adjusted to pH 7,0 with NaOH], the material was covered by a Tris/ferricyanate/X-Gluc solution [9,8 ml Tris buffer + 0,2 ml ferricyanate stock (0,33 g Potassium ferricyanate (Sigma P3667) in 10 ml Tris buffer) + 0,2 ml X-Gluc stock (26,1 mg X-Gluc (Europa Bioproducts ML 113A) in 500 μl DMSO)]. Vacuum infiltration was applied for 15 to 30 minutes. The plants or plant parts were incubated for up to 16 hours at 37 °C until development of blue colour was visible. The samples were washed 3 times for 5 minutes with Tris buffer. Chlorophyll was extracted in ethanol series of 50%, 70% and 90% (each for 30 minutes).

SEQUENCE LISTING

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Claims

1. Method for increasing plant seed yield, comprising introducing into a plant a nucleic acid encoding a cyclin D3 under the control of a promoter capable of preferentially expressing said nucleic acid in the cell expansion zone of a shoot.

2. Method according to claim 1, wherein said increased seed yield is selected from the group consisting of (i) increased

seed biomass; (ii) increased number of (filled) seeds; (iii) increased seed size; (iv) increased seed volume; (v) increased harvest index; and (vi) increased thousand kernel weight.

3. Method according to claim 1 or 2, wherein said nucleic acid encoding a cyclin D3 is obtained from a plant.

4. Method according to any one of claims 1 to 3, wherein said cyclin D3 comprises one or more and preferably all of the following: (i) a cyclin box; (ii) an LxCxE motif within the first 40 or so amino acids; and (iii) one or more and preferably all of the conserved regions identified by the boxes shown in Figure 2 (with one mismatch within the boxes allowed).

5. Method according to any one of claims 1 to 4, wherein said cyclin D3 is encoded by any one of the nucleic acids listed in Table 1.

6. Method according to any one of claims 1 to 5, wherein said cyclin D3 is a homologue having in increasing order of preference at least 80%, 85%, 90%, 95% sequence identity to the amino acid sequence as represented by SEQ ID NO: 2.

7. Method according to any one of claims 1 to 6, wherein said cyclin D3 is as represented by SEQ ID NO: 2.

8. Method according to any one of claims 1 to 7, comprising introducing into a plant a nucleic acid encoding a cyclin D3, which nucleic acid is selected from:

(i) portions of a cyclin D3-encoding nucleic acid;
(ii) alternative splice variants of a cyclin D3-encoding nucleic acid; and
(iii) allelic variants of a cyclin D3-encoding nucleic acid; and

wherein said portions, alternative splice variants, and allelic variants of a cyclin D3-encoding nucleic acid are capable of binding and activating a plant CDK.

9. Method according to any one of claims 1 to 8, wherein said promoter capable of preferentially expressing said nucleic acid in the cell expansion zone of a shoot has a comparable expression profile to a beta-expansin promoter.

10. Method for the production of a transgenic plant having increased seed yield relative to corresponding wild type plants, which method comprises:

(i) introducing into a plant or plant cell a cyclin D3-encoding nucleic acid, preferably a cyclin D3-encoding nucleic acid as represented by SEQ ID NO: 1, which nucleic acid encodes a cyclin D3 polypeptide, which polypeptide is preferably as represented by SEQ ID NO: 2 and which nucleic acid is operably linked to a promoter capable of preferentially expressing said nucleic acid in the cell expansion zone of a shoot;
(ii) cultivating the plant cell under conditions promoting regeneration and mature plant growth.

11. Method according to claim 10, wherein said increased seed yield is selected from one or more of: (i) increased seed biomass; (ii) increased number of (filled) seeds; (iii) increased seed size; (iv) increased seed volume; (v) increased harvest index; and (iv) increased thousand kernel weight,

12. Use of an isolated cyclin D3-encoding nucleic acid operably linked to a promoter capable of preferentially expressing said nucleic acid in the cell expansion zone of a shoot, in increasing seed yield.

13. Use according to claim 12, wherein said seed yield includes any one or more of: (i) increased seed biomass; (ii) increased number of (filled) seeds; (iii) increased seed size; (iv) increased seed volume; (v) increased harvest index; and (iv) increased thousand kernel weight.

14. Use according to claim 12 or 13, wherein said cyclin D3-encoding nucleic acid is a nucleic acid as represented by SEQ ID NO: 1, or wherein said cyclin D3 is an amino acid as represented by SEQ ID NO: 2.

Patentansprüche

1. Verfahren zum Erhöhen des Samenertrags einer Pflanze, umfassend das Einführen einer Nukleinsäure, die für ein Cyclin D3 unter der Kontrolle eines Promoters, der fähig ist, die Nukleinsäure vorzugsweise in der Zellstreckungszone eines Sprosses zu exprimieren, kodiert, in eine Pflanze.

2. Verfahren nach Anspruch 1, wobei der erhöhte Samenertrag aus der Gruppe (i) erhöhte Samenbiomasse, (ii) erhöhte Anzahl (gefüllter) Samen, (iii) erhöhte Samengröße, (iv) erhöhtes Samenvolumen, (v) erhöhter Harvest Index und (vi) erhöhtes Tausendkorngewicht ausgewählt ist.

3. Verfahren nach Anspruch 1 oder 2, wobei die Nukleinsäure, die für ein Cyclin D3 kodiert, von einer Pflanze stammt.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei das Cyclin D3 eines oder mehrere, vorzugsweise alle, der folgenden Elemente umfaßt: (i) eine Cyclin-Box, (ii) ein LxCxE-Motiv innerhalb der ersten ungefähr 40 Aminosäuren, und (iii) eine oder mehrere, vorzugsweise alle, der konservierten Regionen, die durch die in Abbildung 2 angegebenen Boxen identifiziert sind (wobei ein Mismatch innerhalb der möglichen Boxen gestattet ist).

5. Verfahren nach einem der Ansprüche 1 bis 4, wobei das Cyclin D3 von einer beliebigen der in Tabelle 1 angeführten Nukleinsäuren kodiert wird.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei es sich bei dem Cyclin D3 um ein Homolog mit mit ansteigender Bevorzugung mindestens 80%, 85%, 90%, 95% Sequenzidentität zu der Aminosäuresequenz gemäß SEQ ID NO: 2 handelt.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Cyclin D3 gemäß SEQ ID NO: 2 dargestellt ist.

8. Verfahren nach einem der Ansprüche 1 bis 7, umfassend das Einführen einer Nukleinsäure, die für ein Cyclin D3 kodiert, in eine Pflanze, wobei die Nukleinsäure aus der Reihe:

   (i) Abschnitte einer Cyclin-D3-Kodiernukleinsäure,
   (ii) alternative Spleißvarianten einer CyclinD3-Kodiernukleinsäure, und
   (iii) Allelvarianten einer Cyclin-D3-Kodiernukleinsäure

   Ausgewählt ist, und
   wobei die Abschnitte, alternativen Spleißvarianten und Allelvarianten einer CyclinD3-Kodiernukleinsäure fähig sind, eine pflanzliche CDK zu binden und zu aktivieren.

9. Verfahren nach einem der Ansprüche 1 bis 8, wobei der Promoter, der fähig ist, die Nukleinsäure vorzugsweise in der Zellstreckungszone eines Sprosses zu exprimieren, ein Expressionsprofil aufweist, das mit einem beta-Expansin-Promoter vergleichbar ist.

10. Verfahren zur Erzeugung einer transgenen Pflanze mit im Vergleich zu entsprechenden Wildtyppflanzen erhöhtem Samenertrag, wobei das Verfahren folgendes umfaßt:

    (i) Einführen einer Cyclin-D3-Kodiernukleinsäure, vorzugsweise einer Cyclin-D3-Kodiernukleinsäure gemäß SEQ ID NO: 1, in eine Pflanze oder Pflanzenzelle, wobei die Nukleinsäure für ein Cylcin-D3-Polypeptid kodiert, welches vorzugsweise gemäß SEQ ID NO: 2 ist und wobei die Nukleinsäure operativ mit einem Promoter, der fähig ist, die Nukleinsäure vorzugsweise in der Zellstreckungszone eines Sprosses zu exprimieren, verbunden ist;
    (ii) Kultivieren der Pflanzenzelle unter Bedingungen, die Regeneration und das Wachstum einer reifen Pflanze fordern.

11. Verfahren nach Anspruch 10, wobei der erhöhte Samenertrag aus der Gruppe (i) erhöhte Samenbiomasse, (ii) erhöhte Anzahl (gefüllter) Samen, (iii) erhöhte Samengröße, (iv) erhöhtes Samenvolumen, (v) erhöhter Harvest Index und (vi) erhöhtes Tausendkorngewicht ausgewählt ist.

12. Verwendung einer isolierten Cyclin-D3-Kodiernukleinsäure, die mit einem Promoter, der fähig ist, die Nukleinsäure vorzugsweise in der Zellstreckungszone eines Sprosses zu exprimieren, operativ verbunden ist, zum Erhöhen des

Samenertrags.

13. Verwendung nach Anspruch 12, wobei der Samenertrag eines oder mehrere der folgenden Elemente beinhaltet: (i) erhöhte Samenbiomasse, (ii) erhöhte Anzahl (gefüllter) Samen, (iii) erhöhte Samengröße, (iv) erhöhtes Samenvolumen, (v) erhöhter Harvest Index und (vi) erhöhtes Tausendkorngewicht.

14. Verwendung nach Anspruch 12 oder 13, wobei es sich bei der Cyclin-D3-Kodiernukleinsäure um eine Nukleinsäure gemäß SEQ ID NO: 1 handelt bzw. wobei es sich bei dem Cyclin D3 um eine Aminosäure gemäß SEQ ID NO: 2 handelt.

Revendications

1. Procédé pour augmenter le rendement en grains d'une plante, comprenant l'introduction dans une plante d'un acide nucléique codant pour une cycline D3 sous le contrôle d'un promoteur permettant d'exprimer préférentiellement ledit acide nucléique dans la zone d'expansion cellulaire d'une pousse.

2. Procédé selon la revendication 1, ledit rendement en grains accru étant choisi dans le groupe constitué par (i) une biomasse des grains accrue ; (ii) un nombre accru de grains (pleins) ; (iii) une taille des grains accrue ; (iv) un volume de grains accru ; (v) un indice de moisson accru ; et (vi) un poids de mille grains accru.

3. Procédé selon la revendication 1 ou 2, ledit acide nucléique codant pour une cycline D3 étant obtenu à partir d'une plante.

4. Procédé selon l'une quelconque des revendications 1 à 3, ladite cycline D3 comprenant un ou plusieurs et de préférence la totalité des éléments suivants : (i) une boîte cycline ; (ii) un motif LxCxE dans environ les 40 premiers acides aminés ; et (iii) une ou plusieurs et de préférence la totalité des régions conservées identifiées par les boîtes indiquées dans la Figure 2 (avec un mésappariement dans les boîtes permis).

5. Procédé selon l'une quelconque des revendications 1 à 4, ladite cycline D3 étant codée par l'un quelconque des acides nucléiques listés dans le Tableau 1.

6. Procédé selon l'une quelconque des revendications 1 à 5, ladite cycline D3 étant un homologue ayant dans l'ordre croissant de préférence une identité de séquence d'au moins 80 %, 85 %, 90 %, 95 % par rapport à la séquence d'acides aminés telle que représentée par SEQ ID n° : 2.

7. Procédé selon l'une quelconque des revendications 1 à 6, ladite cycline D3 étant telle que représentée par SEQ ID n° : 2.

8. Procédé selon l'une quelconque des revendications 1 à 7, comprenant l'introduction dans une plante d'un acide nucléique codant pour une cycline D3, lequel acide nucléique est choisi parmi :

(i) des parties d'un acide nucléique codant pour une cycline D3 ;
(ii) des variants d'épissage alternatif d'un acide nucléique codant pour une cycline D3 ; et
(iii) des variants alléliques d'un acide nucléique codant pour une cycline D3 ; et

lesdites parties, lesdits variants d'épissage alternatif et lesdits variants alléliques d'un acide nucléique codant pour une cycline D3 étant aptes à se fixer à une CDK de plante et à activer celle-ci.

9. Procédé selon l'une quelconque des revendications 1 à 8, ledit promoteur permettant d'exprimer préférentiellement ledit acide nucléique dans la zone d'expansion cellulaire d'une pousse ayant un profil d'expression comparable à celui d'un promoteur bêta-expansine.

10. Procédé pour la production d'une plante transgénique ayant un rendement en grains accru par rapport aux plantes de type sauvage correspondantes, lequel procédé comprend :

(i) l'introduction dans une plante ou une cellule végétale d'un acide nucléique codant pour une cycline D3, de préférence d'un acide nucléique codant pour une cycline D3 tel que représenté par SEQ ID n° : 1, lequel acide

nucléique code pour un polypeptide cycline D3, lequel polypeptide est de préférence tel que représenté par SEQ ID n° : 2 et lequel acide nucléique est lié de façon fonctionnelle à un promoteur permettant d'exprimer préférentiellement ledit acide nucléique dans la zone d'expansion cellulaire d'une pousse ;
(ii) la culture de la cellule végétale dans des conditions favorisant la régénération et la croissance d'une plante mature.

11. Procédé selon la revendication 10, ledit rendement en grains accru étant choisi parmi un ou plusieurs éléments parmi : (i) une biomasse des grains accrue ; (ii) un nombre accru de grains (pleins) ; (iii) une taille des grains accrue ; (iv) un volume de grains accru ; (v) un indice de moisson accru ; et (vi) un poids de mille grains accru.

12. Utilisation d'un acide nucléique codant pour une cycline D3 isolé lié de façon fonctionnelle à un promoteur permettant d'exprimer préférentiellement ledit acide nucléique dans la zone d'expansion cellulaire d'une pousse, dans l'augmentation du rendement en grains.

13. Utilisation selon la revendication 12, ledit rendement en grains comprenant un ou plusieurs éléments quelconques parmi : (i) une biomasse des grains accrue ; (ii) un nombre accru de grains (pleins) ; (iii) une taille des grains accrue ; (iv) un volume de grains accru ; (v) un indice de moisson accru ; et (vi) un poids de mille grains accru.

14. Utilisation selon la revendication 12 ou 13, ledit acide nucléique codant pour une cycline D3 étant un acide nucléique tel que représenté par SEQ ID n° : 1 ou ladite cycline D3 étant un acide aminé tel que représenté par SEQ ID n° : 2.

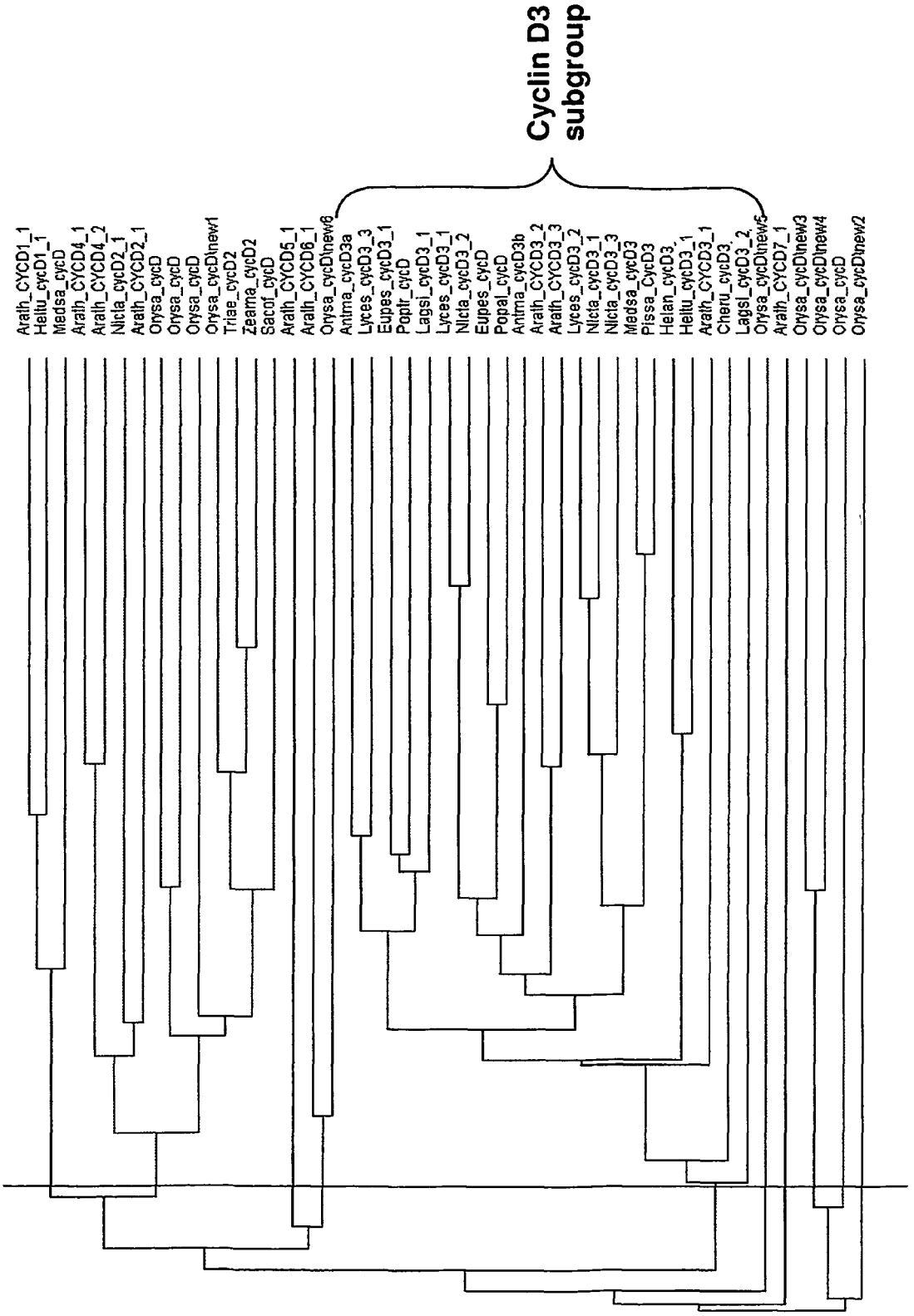

Cyclin D3 subgroup
Arath_CYCD1_1
Heltu_cycD1_1
Medsa_cycD
Arath_CYCD4_1
Arath_CYCD4_2
Nicta_cycD2_1
Arath_CYCD2_1
Orysa_cycD
Orysa_cycD
Orysa_cycDnew1
Triae_cycD2
Zeama_cycD2
Sacof_cycD
Arath_CYCD5_1
Arath_CYCD6_1
Orysa_cycDnew6
Antma_cycD3a
Lyces_cycD3_3
Eupes_cycD3_1
Poptr_cycD
Lagsi_cycD3_1
Lyces_cycD3_1
Nicta_cycD3_2
Eupes_cycD
Popal_cycD
Antma_cycD3b
Arath_CYCD3_2
Arath_CYCD3_3
Lyces_cycD3_2
Nicta_cycD3_1
Nicta_cycD3_3
Medsa_cycD3
Pissa_CycD3
Helan_cycD3_
Heltu_cycD3_1
Arath_CYCD3_1
Cheru_cycD3_
Lagsi_cycD3_2
Orysa_cycDnew5
Arath_CYCD7_1
Orysa_cycDnew3
Orysa_cycDnew4
Orysa_cycD
Orysa_cycDnew2

FIGURE 1

FIGURE 2

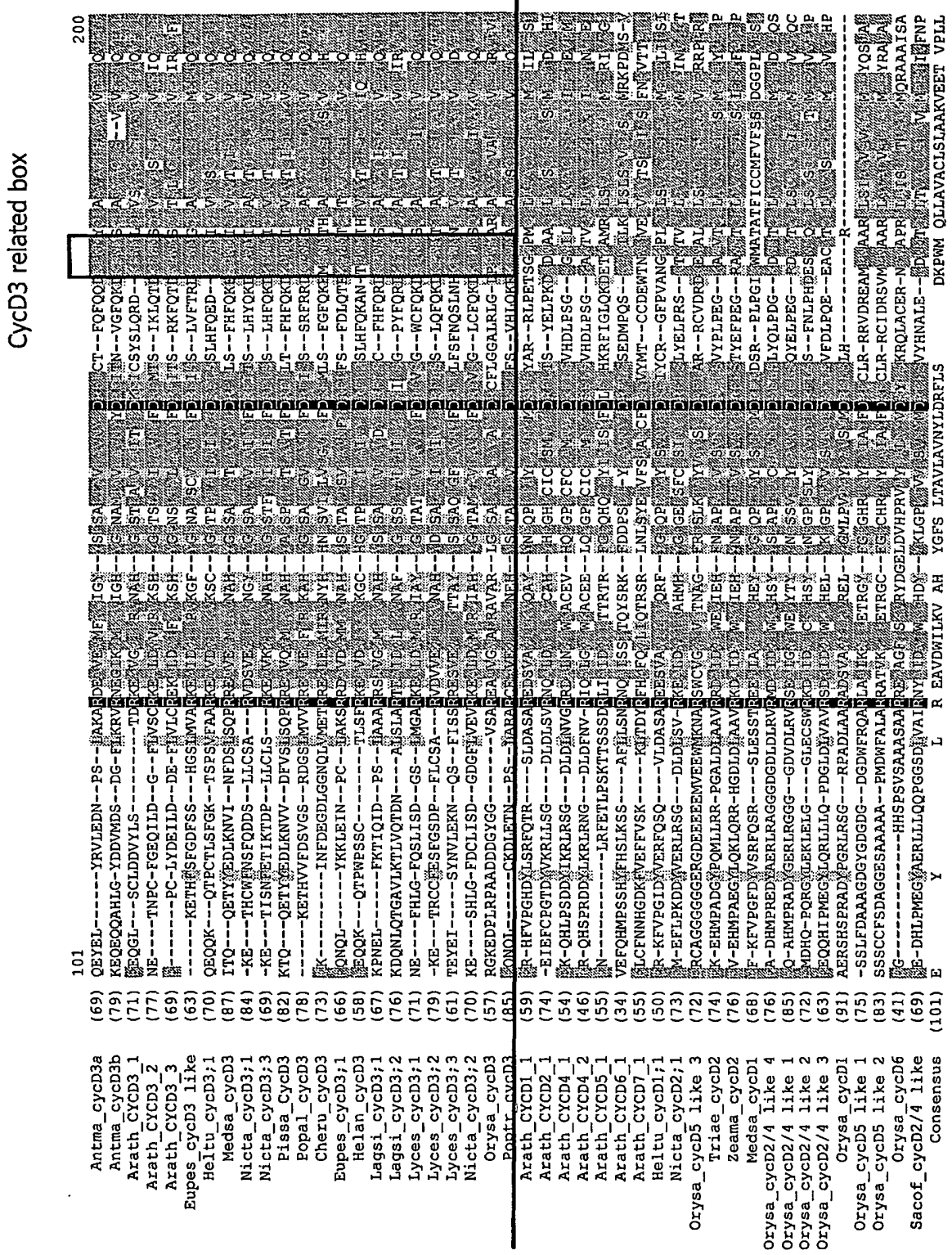


FIGURE 2 (continued)

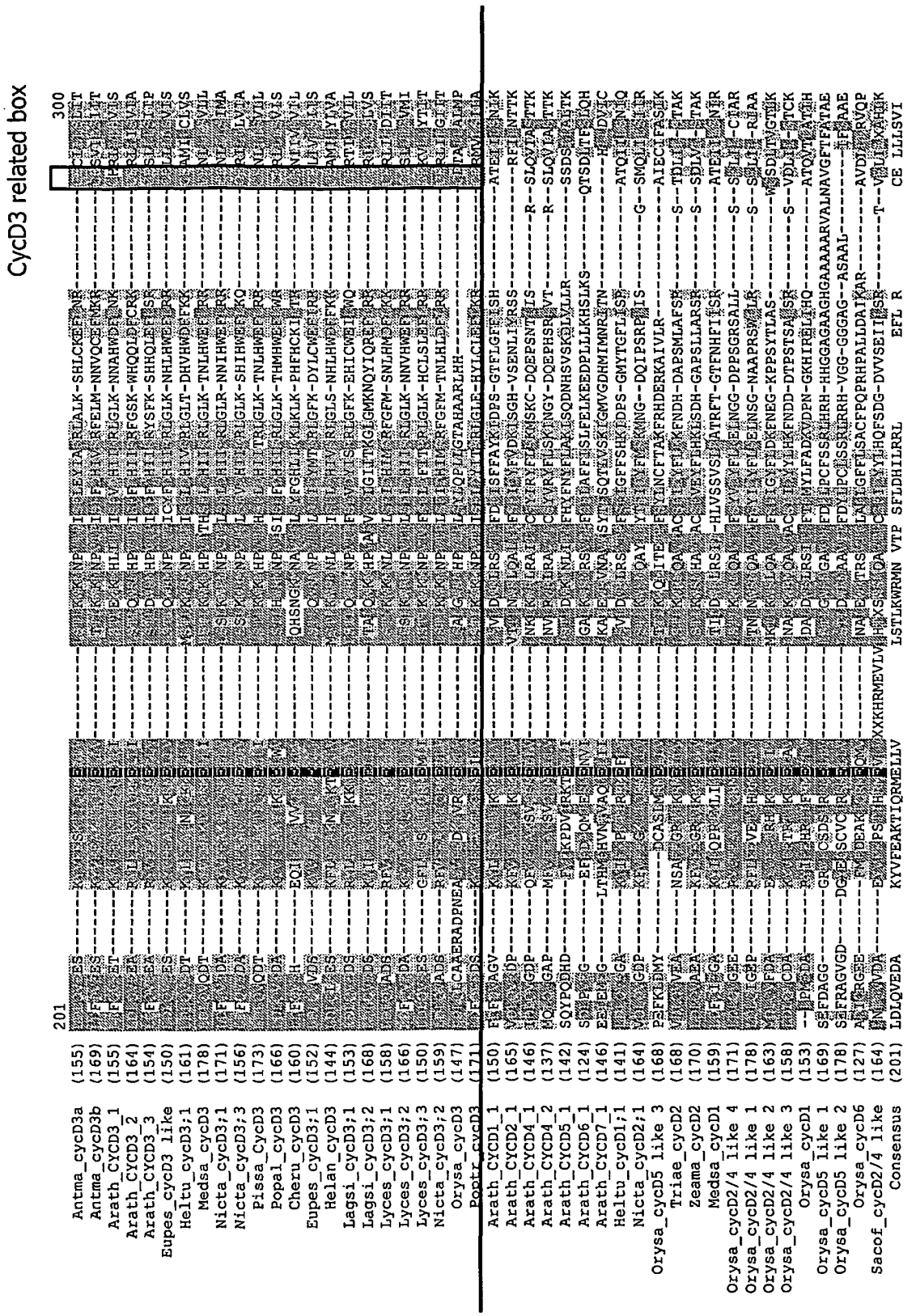


FIGURE 2 (continued)

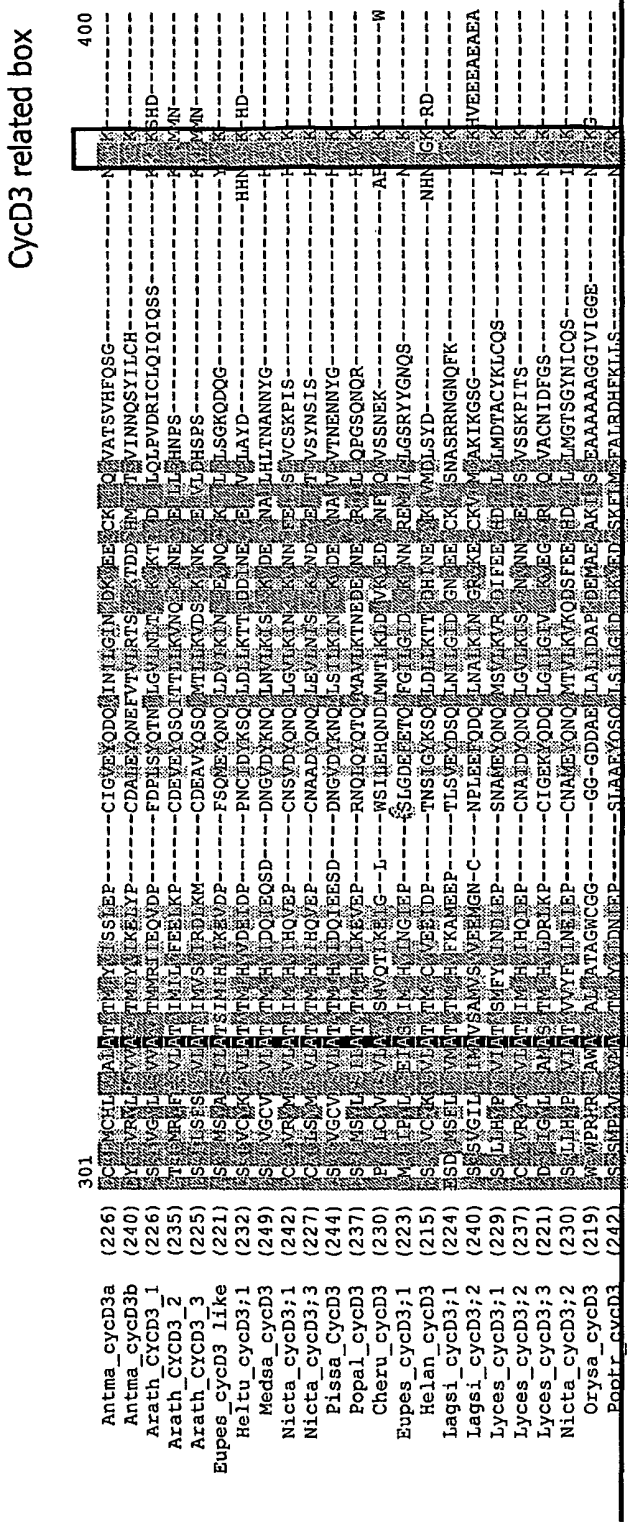


FIGURE 2 (continued)

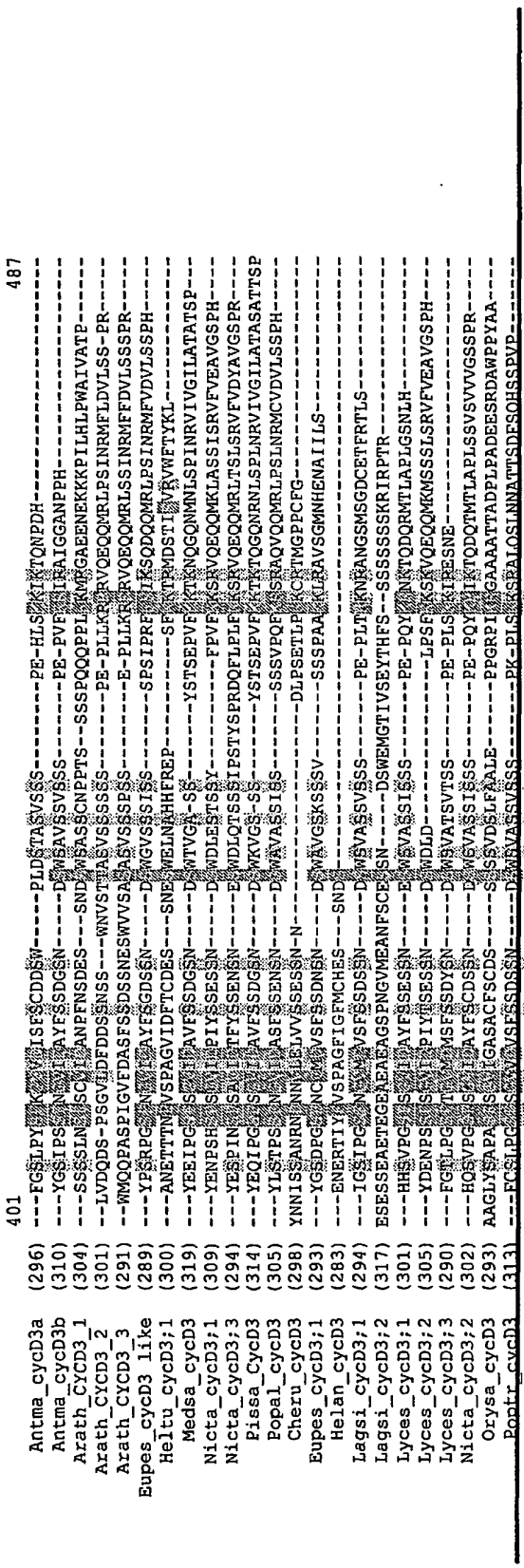
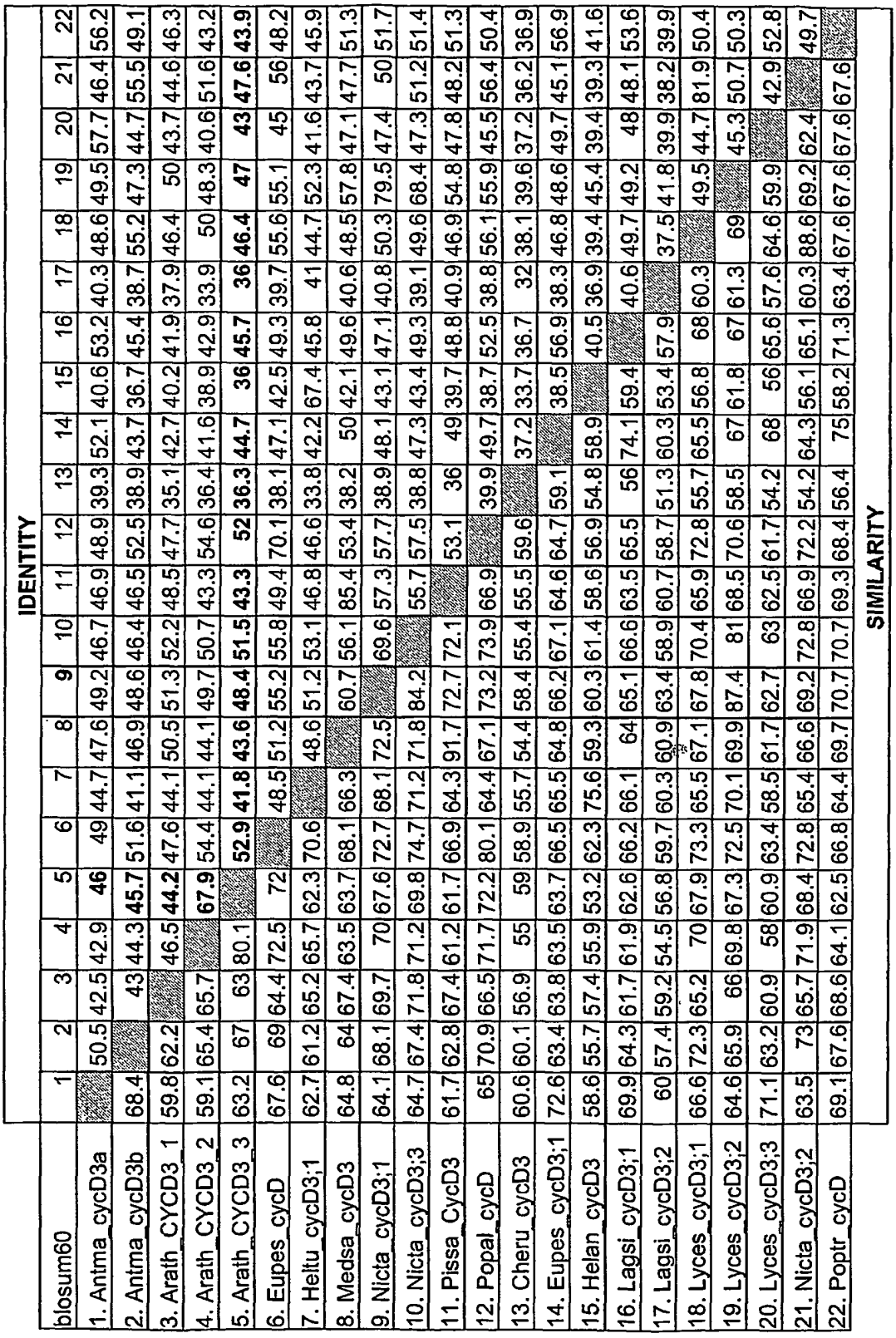


FIGURE 2 (continued)



IDENTITY																						
blosum60	1	2	3	4	5	6	7	8	**9**	10	11	12	13	14	15	16	17	18	19	20	21	22
1. Antma_cycD3a		50.5	42.5	42.9	**46**	49	44.7	47.6	49.2	46.7	46.9	48.9	39.3	52.1	40.6	53.2	40.3	48.6	49.5	57.7	46.4	56.2
2. Antma_cycD3b	68.4		43	44.3	**45.7**	51.6	41.1	46.9	48.6	46.4	46.5	52.5	38.9	43.7	36.7	45.4	38.7	55.2	47.3	44.7	55.5	49.1
3. Arath_CYCD3_1	59.8	62.2		46.5	**44.2**	47.6	44.1	50.5	51.3	52.2	48.5	47.7	35.1	42.7	40.2	41.9	37.9	46.4	50	43.7	44.6	46.3
4. Arath_CYCD3_2	59.1	65.4	65.7		**67.9**	54.4	44.1	44.1	49.7	50.7	43.3	54.6	36.4	41.6	38.9	42.9	33.9	50	48.3	40.6	51.6	43.2
5. Arath_CYCD3_3	63.2	67	63	80.1		**52.9**	**41.8**	**43.6**	**48.4**	**51.5**	**43.3**	**52**	**36.3**	**44.7**	**36**	**45.7**	**36**	**46.4**	**47**	**43**	**47.6**	**43.9**
6. Eupes_cycD	67.6	69	64.4	72.5	72		48.5	51.2	55.2	55.8	49.4	70.1	38.1	47.1	42.5	49.3	39.7	55.6	55.1	45	56	48.2
7. Heltu_cycD3;1	62.7	61.2	65.2	65.7	62.3	70.6		48.6	51.2	53.1	46.8	46.6	33.8	42.2	67.4	45.8	41	44.7	52.3	41.6	43.7	45.9
8. Medsa_cycD3	64.8	64	67.4	63.5	63.7	68.1	66.3		60.7	56.1	85.4	53.4	38.2	50	42.1	49.6	40.6	48.5	57.8	47.1	47.7	51.3
9. Nicta_cycD3;1	64.1	68.1	69.7	70	67.6	72.7	68.1	72.5		69.6	57.3	57.7	38.9	48.1	43.1	47.1	40.8	50.3	79.5	47.4	50	51.7
10. Nicta_cycD3;3	64.7	67.4	71.8	71.2	69.8	74.7	71.2	71.8	84.2		55.7	57.5	38.8	47.3	43.4	49.3	39.1	49.6	68.4	47.3	51.2	51.4
11. Pissa_CycD3	61.7	62.8	67.4	61.2	61.7	66.9	64.3	91.7	72.7	72.1		53.1	36	49	39.7	48.8	40.9	46.9	54.8	47.8	48.2	51.3
12. Popal_cycD	65	70.9	66.5	71.7	72.2	80.1	64.4	67.1	73.2	73.9	66.9		39.9	49.7	38.7	52.5	38.8	56.1	55.9	45.5	56.4	50.4
13. Cheru_cycD3	60.6	60.1	56.9	55	59	58.9	55.7	54.4	58.4	55.4	55.5	59.6		37.2	33.7	36.7	32	38.1	39.6	37.2	36.2	36.9
14. Eupes_cycD3;1	72.6	63.4	63.8	63.5	63.7	66.5	65.5	64.8	66.2	67.1	64.6	64.7	59.1		38.5	56.9	38.3	46.8	48.6	49.7	45.1	56.9
15. Helan_cycD3	58.6	55.7	57.4	55.9	53.2	62.3	75.6	59.3	60.3	61.4	58.6	56.9	54.8	58.9		40.5	36.9	39.4	45.4	39.4	39.3	41.6
16. Lagsi_cycD3;1	69.9	64.3	61.7	61.9	62.6	66.2	66.1	64	65.1	66.6	63.5	65.5	56	74.1	59.4		40.6	49.7	49.2	48	48.1	53.6
17. Lagsi_cycD3;2	60	57.4	59.2	54.5	56.8	59.7	60.3	60.9	63.4	58.9	60.7	58.7	51.3	60.3	53.4	57.9		37.5	41.8	39.9	38.2	39.9
18. Lyces_cycD3;1	66.6	72.3	65.2	70	67.9	73.3	65.5	67.1	67.8	70.4	65.9	72.8	55.7	65.5	56.8	68	60.3		49.5	44.7	81.9	50.4
19. Lyces_cycD3;2	64.6	65.9	66	69.8	67.3	72.5	70.1	69.9	87.4	81	68.5	70.6	58.5	67	61.8	67	61.3	69		45.3	50.7	50.3
20. Lyces_cycD3;3	71.1	63.2	60.9	58	60.9	63.4	58.5	61.7	62.7	63	62.5	61.7	54.2	68	56	65.6	57.6	64.6	59.9		42.9	52.8
21. Nicta_cycD3;2	63.5	73	65.7	71.9	68.4	72.8	65.4	66.6	69.2	72.8	66.9	72.2	54.2	64.3	56.1	65.1	60.3	88.6	69.2	62.4		49.7
22. Poptr_cycD	69.1	67.6	68.6	64.1	62.5	66.8	64.4	69.7	70.7	70.7	69.3	68.4	56.4	75	58.2	71.3	63.4	67.6	67.6	67.6	67.6	

SIMILARITY

FIGURE 3

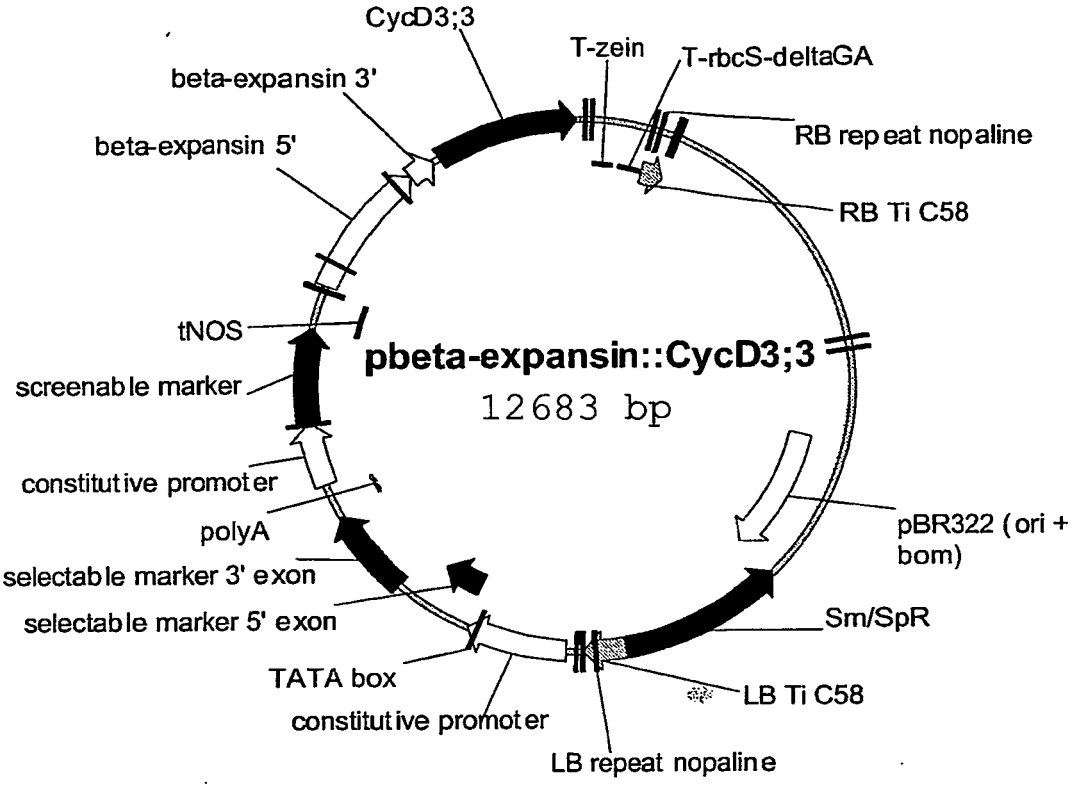
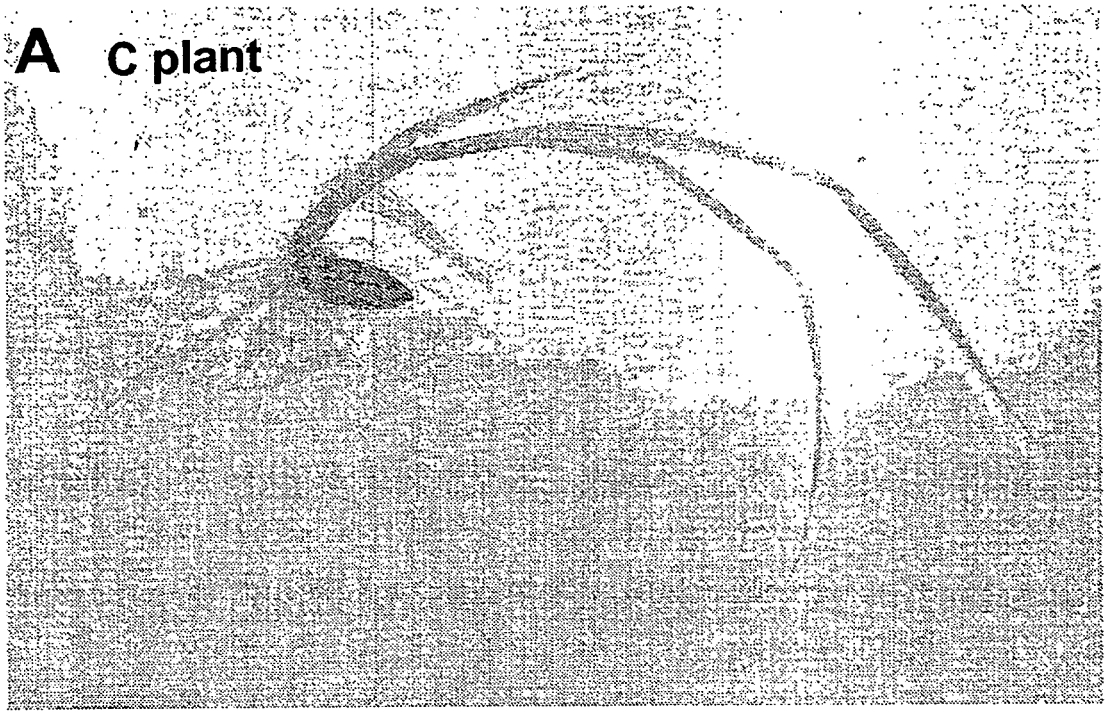

CycD3;3
T-zein
T-rbcS-deltaGA
beta-expansin 3'
RB repeat nopaline
beta-expansin 5'
RB Ti C58
tNOS
pbeta-expansin::CycD3;3
12683 bp
screenable marker
constitutive promoter
pBR322 (ori + bom)
polyA
selectable marker 3' exon
selectable marker 5' exon
Sm/SpR
TATA box
LB Ti C58
constitutive promoter
LB repeat nopaline

FIGURE 4



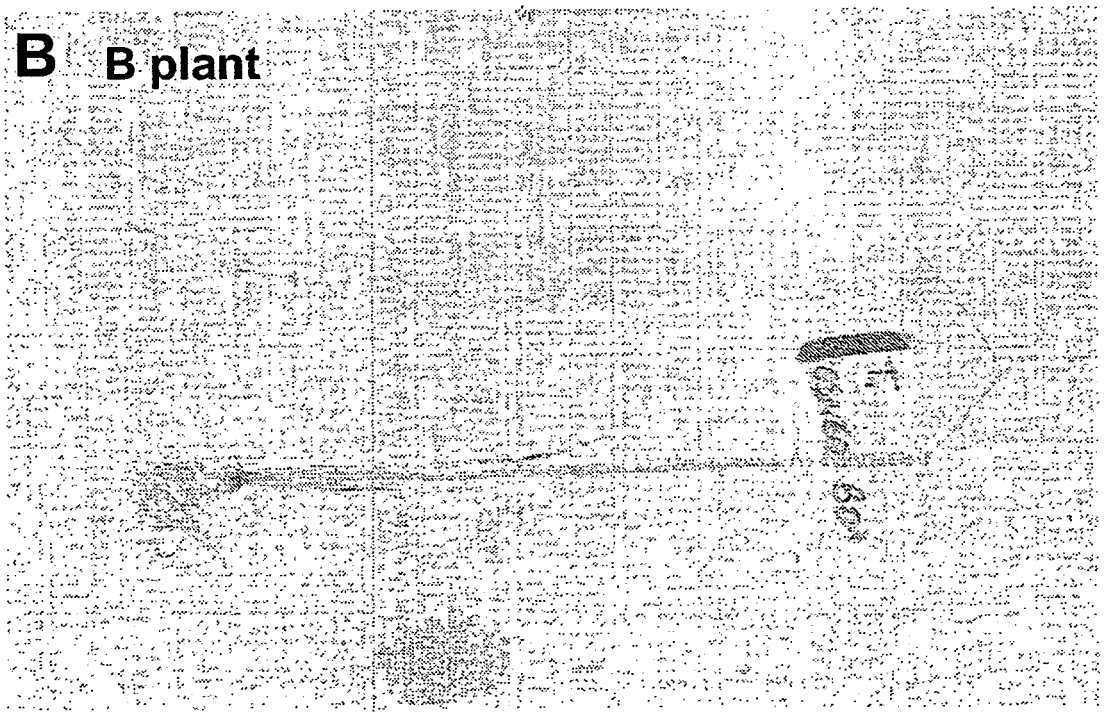


FIGURE 5

SEQ ID NO 1: cyclin D3;3 cDNA

atggctttagaagaggaggaagagagtcaaaacgcaccgttttgtgttcttgatggtct
tttctgtgaggaagagagtgagtttcacgaacaagtagatttgtgcgacgagagtgttg
aaaagtttccttttttaaatctgggtttgtctgatcatgatatgttgtgggatgatgat
gagttatcaactttgatttcgaaacaagaaccgtgtctttatgacgaaatcttagatga
tgagtttctggttttgtgtcgtgaaaaggctcttgattggatttttaaagtgaaatctc
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acaagcaggaagtttcagacagataagccatggatgtctcagcttactgctttggcttg
tctgtctttagctgctaaggttgaagagatccgtgttccttttctcttagattttcaag
tggaagaagcaagatatgtctttgaagctaagactatacagagaatggagcttcttgtt
ctgtctactcttgactggaggatgcatcctgtgactccaatctcgttttcgatcacat
tattcgacgatacagctttaaatctcatcatcaattggagttcttgagtagatgtgaat
ctttattactctccattattcctgattcgagatttctgagttttagtccttctgtgtta
gccactgcaataatggtctctgttattagagatttgaagatgtgtgacgaagctgtata
ccaatctcagctcatgactctactcaaagttgattcggagaaggtaaataaatgctatg
agttagtgttagaccacagtccaagcaagaaaaggatgatgaattggatgcaacaaccc
gctagtccgatcggtgtgtttgatgcgtcattcagttctgatagctcgaatgagtcgtg
ggttgtgtctgcttctgcttcagtgtcgtcttcaccatcttcagagcctttgctcaaga
ggagaagagtgcaagagcagcagatgaggctatcttcaataaaccgaatgtttttcgat
gtgcttagtagtagtcctcgctaa

SEQ ID NO 2: cyclin D3;3 protein

MALEEEEESQNAPFCVLDGLFCEEESEFHEQVDLCDESVEKFPFLNLGLSDHDMLWDDD
ELSTLISKQEPCLYDEILDDEFLVLCREKALDWIFKVKSHYGFNSLTALLAVNYFDRFI
TSRKFQTDKPWMSQLTALACLSLAAKVEEIRVPFLLDFQVEEARYVFEAKTIQRMELLV
LSTLDWRMHPVTPISFFDHIIRRYSFKSHHQLEFLSRCESLLLSIIPDSRFLSFSPSVL
ATAIMVSVIRDLKMCDEAVYQSQLMTLLKVDSEKVNKCYELVLDHSPSKKRMMNWMQQP
ASPIGVFDASFSSDSSNESWVVSASASVSSSPSSEPLLKRRRVQEQQMRLSSINRMFFD
VLSSSPR

FIGURE 6

SEQ ID NO 3: beta-expansin promoter of *Oryza sativa*

aaaaccaccgagggacctgatctgcaccggttttgatagttgagggacccgttgtgtct
ggttttccgatcgagggacgaaaatcggattcggtgtaaagttaagggacctcagatga
acttattccggagcatgattgggaagggaggacataaggcccatgtcgcatgtgtttgg
acggtccagatctccagatcactcagcaggatcggccgcgttcgcgtagcacccgcggt
ttgattcggcttcccgcaaggcggcggccggtggccgtgccgccgtagcttccgccgga
agcgagcacgccgccgccgccgacccggctctgcgtttgcaccgccttgcacgcgatac
atcgggatagatagctactactctctccgtttcacaatgtaaatcattctactattttc
cacattcatattgatgttaatgaatatagacatatatatctatttagattcattaacat
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tacctacgatggatggatgcaggatcatgaaagaattaatgcaagatcgtatctgccgc
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ggaaaagactcaacattaatcaccaaatatttcgccttctccagcagaatatatatctc
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tgtcgtctcaccgtcgcacactggccttccatctcaggctagctttctcagccacccat
cgtacatgtcaactcggcgcgcgcacaggcacaaattacgtacaaaacgcatgaccaaa
tcaaaaccaccggagaagaatcgctcccgcgcgcggcggcggcgcgcacgtacgaatgc
acgcacgcacgcccaaccccacgacacgatcgcgcgcgacgccggcgacaccggccatc
cacccgcgccctcacctcgccgactataaatacgtaggcatctgcttgatcttgtcatc
catctcaccaccaaaaaaaaaggaaaaaaaaacaaaacacaccaagccaaataaaagcg
acaa

SEQ ID NO 4: primer prm0360

ggggacaagtttgtacaaaaaagcaggcttcacaatggctttagaagaggagga

SEQ ID NO 5: primer prm0361

ggggaccactttgtacaagaaagctgggtttagcgaggactactataagca

FIGURE 6 (continued)

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- EP 1198985 A1 **[0067]**
- EP 04100991 A **[0109]**
- US 60553418 B **[0109]**

Non-patent literature cited in the description

- **Healy et al.** *J. Biol. Chem.,* 2001, vol. 276 (10), 7041-7047 **[0030]**
- **Swaminathan et al.** *Plant Phys,* 2000, vol. 124, 1658-1667 **[0030]**
- **Creighton.** Proteins. W.H. Freeman and Company, 1984 **[0040]**
- **Needleman ; Wunsch.** *J. Mol. Biol.,* 1970, vol. 48, 443-453 **[0050]**
- **Krens, F.A. et al.** *Nature,* 1982, vol. 296, 72-74 **[0067]**
- **Negrutiu I. et al.** *Plant Mol. Biol.,* June 1987, vol. 8, 363-373 **[0067]**
- **Shillito R.D. et al.** *Bio/Technol,* 1985, vol. 3, 1099-1102 **[0067]**
- **Crossway A. et al.** *Mol. Gen Genet,* 1986, vol. 202, 179-185 **[0067]**
- **Klein T.M. et al.** *Nature,* 1987, vol. 327, 70 **[0067]**
- **Aldemita ; Hodges.** *Planta,* 1996, vol. 199, 612-617 **[0067]**
- **Chan et al.** *Plant Mol. Biol.,* 1993, vol. 22 (3), 491-506 **[0067]**
- **Hiei et al.** *Plant J.,* 1994, vol. 6 (2), 271-282 **[0067]**
- **Ishida et al.** *Nat. Biotechnol.,* June 1996, vol. 14 (6), 745-50 **[0067]**
- **Frame.** *Plant Physiol.,* May 2002, vol. 129 (1), 13-22 **[0067]**
- **Hayashi et al.** *Science,* 1992, 1350-1353 **[0074]**
- **McCallum.** *Nat Biotechnol,* April 2000, vol. 18 (4), 455-7 **[0075]**
- **Stemple.** TILLING-a high-throughput harvest for functional genomics. *Nat Rev Genet.,* February 2004, vol. 5 (2), 145-50 **[0075]**
- **Offringa et al.** Extrachromosomal homologous recombination and gene targeting in plant cells after Agrobacterium-mediated transformation. *EMBO J.,* October 1990, vol. 9 (10), 3077-84 **[0078]**
- **Terada R ; Urawa H ; Inagaki Y ; Tsugane K ; Iida S.** Efficient gene targeting by homologous recombination in rice. *Nat Biotechnol.,* 2002 **[0078]**
- **Terada.** A tale of two integrations, transgene and T-DNA: gene targeting by homologous recombination in rice. *Curr Opin Biotechnol.,* April 2004, vol. 15 (2), 132-8 **[0078]**
- **Sambrook.** Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, 2001 **[0088]**
- **Ausubel et al.** Current Protocols in Molecular Biology. 1994, vol. 1, 2 **[0088]**
- Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase. **R.D.D. Croy.** Current Protocols. BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, 1993 **[0088]**